The effects of postconditioning after renal ischaemia-reperfusion in the cases of rat models with metabolic damages

Ph. D. dissertation (theses summary)

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I. INTRODUCTION

Ischaemia-reperfusion (IR) injury frequently threatened not only the treated organ but the whole body and the integrity of the organs during different surgeries. An important component of the successful transplantation is the prevention from damage caused by IR. Renal ischaemia as a consequence of arterial occlusion, shock and organ transplantation is a common cause of renal failure. The arterial and venous occlusion occur together during renal transplantation, when the vessels are artifically blocked. One of the most important and studied risk factors of renal transplantation is the IR damage. Ischaemia-reperfusion injury causes a variety of changes in tissue homeostasis that leads to necrosis and/or programmed cell death. The ischaemia can lead to tubular necrosis. Necrosis of papillary ducts often appears during acute kidney rejection and diabetes mellitus. This accompanied with high level of cytokine formation (TNF-α, IL-1, IL-6, IL-8). These are responsible for the systemic inflammation responses besides the injury of the tubular basal cells. TNF-α, localized mainly in endothelial cells, macrophages and parenchymal cells of kidney, has a main role in kidney damage.

The endogenous adaptation induced by ischaemic pre- and postconditioning is the most effective defense against ischaemia-reperfusion injury ("goldstandard"). Murrey et al. showed 27 years ago in dog-models that short-term ischaemic periods, in a paradox way, decrease the extension of myocardial infarct that occurs later. This phenomenon, where the myocardium reacts with adaptive and protective response to the ischemic stimulus, is called "ischaemic **preconditioning"**. Its usefulness was realised in lung, skeletal muscle, brain, intestines and kidney as well. The efficiency of this procedure was proved in more mammal species. It can be a relative defense due to short-term ischaemic periods or with farmacological procedures. The clinical usage of preconditioning remained limited because the preconditioning must happen before the ischemic attack or it can not have its defense effect.

In 2003 Zhao et al. showed that repetitive ischaemia-reperfusion cycles of blood flow in the early phase of reperfusion have improved the outcome in vital organs. Many studies proved the positive effect of **postconditioning** in renal injury but **metabolic alterations (diabetes mellitus, hypercholesterolaemia)** reduce or abolish the beneficial effects of these manoeuvers. High cholesterol level induces mitochondrial dysfunction and apoptosis in several cell types. The development of endogenous adaptation sustains a loss in hyperlipidaemia and diabetes. The postconditioning remained inefficient in genetically diabetic ob/ob mice after the myocardial infarct and also the activation of kinases, playing a role in protective signalling pathways decreased. So the protective effects of PS could not occur. During the IR the complex inflammation cascade is activated, in the induction and maintenance of these the proinflammatory cytokines (TNF-α) have an important function. The decreased blood flow causes the fall of the protective factors and cytokine release from the tissue, so they reach high concentrations in the damaged tissue. Tumor necrosis factor is a pleiotropic proinflammatory cytokine that exerts multiple biologic effects. TNF-α is one of the most important proinflammatoric cytokines in the pathogenesis of IR damages. TNF-α induces three pathways: apoptotic cell death; activation of MAPK pathway; and NFKB-

pathway. TNF-α has two receptors (TNFR I, TNFR II) and also two main effects: protection and stimulation of cell death. The apoptotic pathway is responsible for cell death, the MAPK and NFKB pathways are responsible for cell protection and inflammation. The reactive oxigen species (ROS) can shift the balance between these two pathways.

II. AIMS

In our research program we studied the ischaemia-reperfusion damage of kidney following the operations in the groups of healthy, hypercholesterolemic and diabetic rats. We aimed to decrease the ischaemia-reperfusion damage due to ischaemic and pharmacological postconditioning.

In the first part of this work our aim is to reach ischeamic postconditioning in the kidney of hypercholesterolemic animals due ichaemia-reperfusion (45 min ischaemia, 120 min reperfusion); to use ischaemic postconditioning after occlusion of renal vascular pedicle; and to examine the amount of occured oxidative stress and inflammatory responses. We described the oxidative stress with the amount of lipidperoxidation. The reperfusion induced inflammatory response was described with the TNF-α level; we measured the change of leukocyte activation with the induced production of ROS; we also studied the ischaemia-reperfusion damages of the kidney tissue.

In the second part of our work the purpose of this study is to reach ischeamic postconditioning in the kidney of rats with diabetes mellitus type 2 due ichaemia-reperfusion (90 min ischaemia, 240 min reperfusion); to measure the effect of ischemic postconditioning in the liver tissue; to measure the levels of serum-glucose, insulin and TNF-α during the oral glucose tolerance test (OGTT); to examine the oxidative stress and inflammatory response in blood, serum and peripheral tissue. We described the oxidative stress with the amount of serumperoxidase. The reperfusion induced inflammatory response was described with the TNF-α level and with the leukocyte-induced production of ROS. We also aimed to measure the level and activation of antioxidant-enzimes (SOD, GSH) and the concentration of malondialdehid, which is known as the indirect marker of the lipidperoxidation.

In the third part of this work our aim is to study the ischeamia-repefusion damage in the kidney of animals with diabetes mellitus type 1 (45 min ischaemia, 120 min reperfusion); to examine the effects of ischaemic and pharmacological postconditioning with insulin. Our aim is to study the damage of the kidney and to decrease the effect of the postconditioning with blocking of the main protective pathway (blocking PI3K/Akt pathway with Wortmannin); and also to study the ischaemia-reperfusion damage in serum and peripheral tissues with the measurement of the TNF-α level.

III. EFFECTS OF POSTCONDITIONING ON KIDNEY ISCHAEMIA-REPERFUSION INJURY IN HYPERCHOLESTEROLEMIC RATS

1. Aims

The purpose of this work is to study the effect of ischaemia-reperfusion damages after 45 minutes of kidney-ischaemia, and 240 minutes of reperfusion in healthy and hypercholesterolemic rats. We used 4 cycles of 15-second-reperfusion and 15-second-reocclusion in the ischeamic postconditioned group in the beginning phase of reperfusion. In this model we wanted to study the inducation of mechanisms of endogene adaptation and the effect of ischaemic postconditioning in blood, serum and kidney tissue. To describe the metabolic status we measured the levels of glucose, cholesterin, trigliceride and insulin; to describe the kidney function the levels of serumcarbamid and creatinin were measured; we described the oxidative stress with the level of lipidperoxidation (serumperoxid-level). The reperfusion induced inflammatory response was described with the TNF-α level and with the leukocyte-induced production of ROS. The effect of the ischaemic postconditioning in renal tissue was shown by light microscopic technics (HE, PAS, TNF-α immunhistochemistry).

2. Materials and methods

2.1. Animal model and diet

Male albino rats of the Wistar strain (250–280 g) were used in the studies from Charles River Breeding Laboratories (Hungary, Isaszeg). Rats were maintained in an air-filtered, temperature-conditioned (25±2ºC) and light-controlled (12 h light-dark cycle) room. Rats were fed with standard commercial pellets and water ad libitum. The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the local institutional Committee on Animal Research of Pecs University (BA No. 02/2000-25/2006.) Rats were divided into four groups (8 rats in each group). The rats in the Two normal feed groups (group NF) were fed with normal rat chow (Altromin GmbH, Germany), and two cholesterol feed groups (group CF) were fed with 1.5% cholesterol containing (Sigma Aldrich) diet for 8 weeks. One normal feed and one cholesterol feed groups underwent an ischaemic-reperfusion (group IR), in addition the other two (one normal and one cholesterol feed) groups were treated with postconditioning after the ischaemic period (group PS). Group NF/IR: normal feed/ ischaemia-reperfusion, Group NF/PS: normal feed/ ischaemic postconditioning, Group CF/IR: cholesterol feed/ ischaemia-reperfusion, Group NF/PS: normal feed/ ischaemic postconditioning.

2.2. Kidney ischaemia-reperfusion model

The animals were anaesthetized with an intraperitoneal injection of ketamine (500 mg ketamine pro ampul) and diazepam (10 mg diazepam pro ampul). The proportion is 1 :1 (0.2 ml/100 gram) and were placed on a pad. Midline laparotomy was applied and the bilateral

renal artery and vein were isolated. After fine isolation of the renal segment, an atraumatic microvascular clamp was placed on the renal vascular pedicle for 45 minutes. Untreated group animals underwent a long ischaemia (45 min.) followed by 120 minutes reperfusion (groups IR). After the ischaemic period, in the other two groups intermittent 15 seconds reperfusion – 15 seconds ischaemic episodes postconditioning were applied four times (groups PS). At the end of the ischaemic period, the clamp was released and blood could be reperfused. The microvascular clamp was removed from the renal vascular pedicle (RVP) and RVP was reperfused for 120 minutes with or without PS protocol.

2.3. Serum cholesterol, triglyceride, carbamide, creatinine levels

Serum cholesterol, triglyceride, creatinine and urea levels were determined by standard photometric methods (Cholesterol-, triglyceride-kit; Diagnosticum Ltd., Hungary).

2.4. ROS detection

ROS was measured in anticoagulated blood immediately after samplings. ROS production in circulating cells (mainly produced by leucocytes) was induced by Phorbol-12-miristate 13-acetate (PMA); 0.2 μg/ml; Sigma-Aldrich Ltd, Budapest) and was made detectable by means of luminol (3.33 μg/ml; (Boehringer Gmbh Mannheim, Germany) and was measured by a lumino-aggregometer (Chrono-log 560-VS; Chrono-log Corp., USA). The maximum values of ROS production were evaluated, by the maximum value of luminescence. Oxidised low density lipoprotein (oxLDL), and tumour necrosis factor alpha (TNF-α), were measured in sera by ELISA kits (R&D Systems Europe Ltd.), according to the user's manual of the kits (Hungarian distributor was Biomedica Hungaria, Budapest).

2.5. Histological examination

The kidney was fixed in 10% neutral-buffered formalin, paraffin-embedded and sectioned 5 μm thick according to the standard procedure. The sections were deparaffinezed and hydrated gradually, and examined by haematoxylin-eosin (HE), perjodic-acidSchiff reaction (PAS) staining. Renal sections were examined in blind fashion for tubular cell swelling, pyknotic nuclei. Damage of papillary ducts often appears during hypoxia (ischaemia) and diabetes mellitus, so we focused on this area. Grading for the proximal tubulus Jablonski-score was used. Jablonski Grading Scale: Score 0 = Normal, Score 1 = Necrosis of individual cells, Score 2 = Necrosis of all cells in adjacent poximal convoluted tubule (PCT) with survival of surrounding tubules, Score 3 = Necrosis confined to distal third of PCT with band(s) of necrosis extending across inner cortex, Score 4 = Necrosis of all 3 segments of PCT.

2.5.1. Immunohistochemistry

Anti-rat TNF-α monoclonal antibody (R&D System, Histopatology Ltd; Pecs) was used. The monoclonal antibody signs the cytoplasma of the TNF-α producing cells. The positive control is the granulomatoid inflammation. TNF-α positivity was evaluated at least 20 microscopic pictures in each slide (magnification: 200×) by MIRAX Viewer program. The

ratio of positive and negative cells were calculated in all case of all field of visions according to the following scoring system: TNF-α in tubular, 20% =Grade 1, 40%= Grade 2, 60% = Grade 3, 80% =Grade 4, 100% = Grade 5.

2.6. Statistical analysis

Results were expressed as mean ± standard error of means (SEM). One-way analysis of variance (ANOVA), Wilcoxon-, Mann-Whitney-test was used. $p< 0.05$ was considered significant.

3. Results

Serum cholesterol, triglyceride, creatinine, urea levels:

Serum cholesterol and triglyceride levels were significantly higher in cholesterol feed rats than in control ones.

Serum creatinine concentrations elevated significantly in each group, in response to the surgical intervention. Above other important roles in the body, urea serves as a carrier of waste nitrogen, and plays a role in the counter current exchange system of the nephrons, that allows reabsorption of water, though it is an important player in the formation of the excreted urine. No differences were observed in groups.

Serum TNF-α quantification:

In the study we measured the serum TNF-α levels before the ischemia and after ischemia-reperfusion in the experimental groups. We have found that in the end of the reperfusion protocol the serum TNF-α concentration was elevated in the normal feed, postconditioned (NF/ PS) group (7,61 ± *2,04* pg/ml vs. 3, 24± *0,77* pg/ml) and in the normal feed, non-conditioned (NF/ IR) group (20± *2,82* pg/ml vs. 6,77± *0,92* pg/ml) groups in comparison to the values before the ischemia. While both in the postconditioned and in the non-conditioned groups the serum TNF-α concentration after the reperfusion was high. In the NF/ PS group the concentration was significantly ($p < 0,05$) lower than in the NF/ IR group (7,61 ± 2,04 pg/ml vs. 20 ± 2,82 pg/ml).

We measured in cholesterol feed, non-conditioned (CF/ IR) group before surgery: 6,53± 2,52 pg/ml, after surgery: 9,39 ± 2,02 pg/ml, in CF/ PS group before surgery: 5,01 ± 1,15 pg/ml, after surgery: 2,77± 1,2 pg/ml. The TNF-α concentration did not increase significantly as an effect of the surgery in the cholesterol feed group .

Histological examination:

Morphological changes were determined in the area of papilla and proximal tubule of the kidney. Cell infiltration and fibrotic disorders could be found. The presence of hyalincylinder was dominant in all parts of the kidneys. The grading of the proximal tubulus based on Jablonski-score. The amount and localisation of hyalincylinder did not show a significant difference in the groups.

Histological detection of TNF-α in reperfused renal tissue: In the area of papilla and in the cytoplasma of tubular epithelium TNF-α accumulation can be presented with microscopic immunohistochemistry.
We counted and statistically calculated the amount of TNF-α in epithelium of papilla. We measured in normal feed, non-conditioned group (NF/ IR) 27,2± 8,03 %. It was significantly higher than in the normal feed, postconditioned group (NF/ PS): 10,8± 1,81%. In response to IR, a significant TNF-α positivity was observed in epithelial cells of the papillary collective ducts, which could not been observed in healthy postconditioned animals. We calculated in cholesterol feed, non-conditioned group (CF/ IR) 44,8± 9,28 % and in CF/ PS group 39,34± 5,67%. Tissue TNF-α level was significantly higher in cholesterol feed group than in normal feed group, and this high level did not change in response to PS. Postconditioning prevented the elevation of tissue TNF-α levels only in healthy animals.

4. Discussion

The postconditioning was publicated at first time by Vinten-Johansen et al.. It was described as the most effective preventing reaction of the body. In experimental environment it was proved that following ischemic during the reperfusion due to the ischemic- reperfusion short time cycle (10-20 seconds) cell and tissue damage was significantly decreased. According to the results of the research programs the method is also used in clinical practice (heart-surgery, vessel-surgery, transplantation). This promising endogenous adaptation has a great affect in healthy organs, but in people suffering from metabolic disease the providence is not, or just partly producable.

We aimed to examine cytokine expression and apoptosis in tissue damage after revascularisation (TNF-α levels in serum and tissue). We studied the protective effects of ischemic postconditioning in healthy rats after ischemia-reperfusion injury. And the purpose of our study was to produce the hypercholesterolemic state, to see if the postconditioning means a protection against reperfusion damages in the case of metabolic disorders. The actuality of our question is proved because during kidney operations the biggest problem is not the acute rejection but the ischemic-reperfusion damage. Lipid abnormalities are frequently associated with renal disease and may trigger renal injury at an early stage. Hypercholesterolaemia (HC) may increase renal oxidative stress, endothelial dysfunction, inflammation, fibrosis and tissue injury. Interestingly, activation of all of the signalling pathways and induction of TNF-α required cholesterol trafficking to the endoplasmic reticulum (ER). HC mediated ER stress leads to the activation of NFKB and MAPK inflammatory signaling pathways.

TNF-α induces three pathways: apoptotic cell death, activation of MAPK pathway and NFKB-pathway. We reached hypercholesterolaemia expression in the high cholesterol fed Wistar male rats. The so created animal models were appropriate to examine the appearance of the induced endogene protection in metabolic disorders too. The amount of IR

damage was described by the level of TNF-α produced by leukocytes and endothel. According to the results of our research in normal feed group the postconditioning reduced the level of TNF-α in serum. The IR damage of hypercholesterolemic animals were moderated compared to the normal animals, but they were not influenced by the following postconditioning.

We could not find differences between our groups but it is possible that after a longer ischemic period, they could be visible. Our hypothesis is that in this early phase we still can not show the general microscopic disorders.

We examined the other light microscopic disorders with immunohistochemical methods. We focused on TNF-α which is the main factor of the inflammation cascade, following the reperfusion. Wilson et al. took an interesting observation: NFKB and TNF-α are highly expressed in the HC kidney and promote renal inflammation. According to our results, TNF-α was detectable in all groups in the cortical and medullar part of the kidney, the most marked differences were in the papilla. We counted and statistically calculated the amount of TNF-α in epithelium of papilla, where we detected significantly lower values in the PS group than in IR group. In the hypercholesterolemic animals the level of TNF-α was significantly higher with and without postconditioning than in healthy animals.

Infiltrating neutrophils and macrophags may exacerbate tissue injury by producing a variety of cytokines. Previous studies have shown that the concentration of the proinflammatory cytokines (TNF-α, IL-6), produced by both macrophags and neutrophils, is elevated in the ischemic kidney and may have a pathophyisiological role following IR injury.

In consequence of our experiments, the postconditioning can reduce the level of TNF-α in healthy organs but in hypercholesterolemic organs's tissue this effect is not developed.

We find it especially important that hypercholesterolemia can increase the production of TNF-α in the tubular cylinder cells of the kidney and in the smooth muscle of vessels in itself.

Our hypothesis is, that the NFKB and MAPK pathways are already induced by HC before the TNF-α receptor-associated pathways. Due to this early response the ischemia won't cause such a significant inflammatoric response. We thought that the HC until a determined level may influence the endogene protection. Rőth et al. found similar results during the study of antioxidant treatment where they also proved that the presence of reactive oxigen species is necessary for activating the endogene antioxidant protection. Our second hypothesis is that HC can be able to induce endogene protection in itself, possibly in a pathway associated with ROS. And this pathway might have a more important role in the serum than in renal tissue. In this way we could explain the differences between the measured TNF-α levels in tissue and in the serum. We need to have further research on these hypothesis.

5. Conclusions

Summerising our results we can establish that the postconditioning, which is an endogen adaptation mechanism, reduces the oxidative stress and the inflammation against the ischemic-reperfusion injury. The clinical importance of this study can be used in the clinical practice in such operations, where firstly hypoxic and ischemic areas are reperfused (heart and vessel surgery, traumatic surgery, surgery of extremities, transplantation). When all is said and done postconditioning seems to be following surgeries an adequate possibility to decrease reperfusion damages, because of its effectivity, speedness and because its simplicity. The success of this method is hardened because many patients suffer from metabolic disorders (hyperlipidaemia, diabetes) and it influences the results a lot. We would like to draw the attention to realize whether postconditioning can be used in just mentioned people.

Further studies are necessary to see detailed the mechanisms in the cases of metabolic disorders and to decide if this method can be used successfully.

IV. EFFECT OF TYPE 2 DIABETES MELLITUS ON GLUCOSE TOLERANCE AND ENDOGENOUS ADAPTATION TO ISCHAEMIA-REPERFUSION KIDNEY INJURY IN RATS

1. Introduction

Type 2 diabetes mellitus is a multifactorial, poligenic disease which is characterized by an improper insulinsecretion, relative lack of insulin and insulin resistance. The fat tissue is also an important regulator of the effect of insulin, because it produces free fatty acids, that help the serin-phosphorilation of insulin-receptor substrate-proteins and so they block the signal pathway of insulin. The fat tissue has also such functions as an endocrin organ: it secrets adipocins, the insulin-antagonist TNF-α, resistin, and the insulin-sensitivity increasing adiponectin and leptin. In the case of insulinresistency it is not able to display the physiologycal effect in organs (skeletal muscle, heart, fat tissue, pancreas, liver), the β-cells of pancreas react with an increased insulinproduction. The diagnosis of T2DM and its preceding conditions can be confirmed by an oral glucose tolerance test (OGTT). If the fasting glucose level is from 5,6 to 6,9 mmol/L, we can talk about impaired fasting glycaemia (IFG). If the 2 hour OGTT glucose level is between 7,8 mmol/L and 11,0mmol/L, confirms the diagnosis of an impaired glucose tolerance (IGT). We talk about Type 2 diabetes mellitus (T2DM) if the random (not fasting) blood glucose level is >11,1 mmol/L, if the fasting glucose level is >7mmol/L or if the 2 hour OGTT level is more than 11,1 mmol/L.

2. Aims

In the second part of this work is our aim to develop ischeamic postconditioning in the kidney of rats with type 2 diabetes mellitus after the occlusion in hilus of the kidney; and to measure the amount of occuring oxidative stress and inflammatory response. Our question is, how do the the levels of serum-glucose, insulin and TNF-α change during the oral glucose tolerance test (OGTT) in normal and T2DM rats.

Our aim is to examine the effect of ischeamic postconditioning in the kidney of rats following 90 minutes of ischemia and 240 minutes of reperfusion. In the group of rats with ischaemic postconditioning we used 4 cycles of 15-second-reperfusion and 15-second-reocclusion in the beginning phase of reperfusion. After that we studied the oxidative stress and inflammatory response in blood, serum and peripheral tissue (liver). We described the oxidative stress with the level of serumperoxid. The reperfusion induced inflammatory response was described with the TNF-α level and with the leukocyte-induced production of ROS. We also measured the level and activation of antioxidant-enzimes (SOD, GSH) and the concentration of malondialdehid, which is known as the indirect marker of the lipidperoxidation.

Our aim was to investigate the effect of high cholesterol level on serum glucose, insulin, cholesterol, triglyceride, and TNF-a level in rats in the course of oral glucose tolerance test. We also aimed to investigate the effect of high cholesterol level on the endogenous adaptation of the rats in the course of renal ischaemia reperfusion injury.

3. Materials and methods

Male Wistar rats (n = 32) were divided into two groups. In group I. the animals were fed with standard rat chow (SC). In group II. the animals were fed with 2 % cholesterol containing chow (CC) for 12 weeks. Food consumption and weight gain were controlled regularly. Oral glucose tolerance test (OGTT) was performed at the 5th and 12th week after 15 hour fasting. 1 g/kg glucose was given orally, and blood samples were taken before and 15, 30, 60 and 120 min after glucose administration. Serum glucose, triglyceride and cholesterol levels were measured by standard photometric, while TNF-a and insulin by ELISA method.

At the end of the 12 week both groups were divided further (n=8 rats each), and under ketamine-xylazine anesthesia, median laparotomy was performed, and both renal pedicles were clamped for 90 minutes, followed by reperfusion for 4 hours, with or without intermittent 15 sec reperfusion and 15 sec ischemic episodes of postconditioning for four times. Serum glucose, insulin, TNF-a levels were determined as before. Phorbol-12-myristate-13 acetate induced free radical generating capacity in whole blood was measured by luminometric method before and after the intervention.

3. Results

Type 2 diabetes mellitus was developed with a diet that contains 2% cholesterol, 0,5% bile-acids and 20% sunflower oil. Fasted TNF-a levels did not differed between groups by the 5th week. TNF-a elevation was higher, and insulin release was lower in cholesterol fed animals. During the oral glucose test it could be demonstrated in rat models that not only insulin release can be observed, but simultaneously, the levels of serum- TNF-α increase too, playing so an important role in the development of insulin resistance. Peripheral insulin resistance was established during aging in metabolically healthy (6 months) Wistar rats. Ischemic postconditioning reduces the ischemia-reperfusion injuries only in level of liver MDA following the 90 minutes of ischemia and 240 minutes of reperfusion. The SOD and glutathione concentration significantly decreased in T2DM after renal ischemia-reperfusion, indicating the usage of them because of increased ROS-production. The concentration of malondialdehyde was significantly increased in the hepatic tissue in group T2DM compared to the healthy control group.

V. EFFECTS OF POSTCONDITIONING ON KIDNEY ISCHAEMIA-REPERFUSION INJURY IN TYPE 1 DIABETES MELLITUS RATS

1. Introduction

Vinten-Johansen et al. publicated in 2005 that it would be a great progress to find a protective pharmacological state which imitates the postconditioning. Many pharmacological ideas can be found in the literature, where different agents were attempted to influence the events of perfusion. Most of these experiments were in connection with the ischaemia-reperfusion injury of myocardial. These were for example adenosine and its analogs, nitrogen monoxide, insulin, opioids, CO_2, and many other materials. So far non of the drugs were implemented in the routine clinical practice. It has not been proved if the intermittent administration of pharmacological agents are able to imitate the time-characteristics of the postconditioning and thus if their application is more effective.

2. Aims

In the third part of our experiments we aim to examine the ischaemic and pharmacological postconditioning in type 1 diabetic animal models. The cardioprotective effect of insulin has been shown during coronary ischaemia. There have not been extensive studies according to the examination of the insulin in the renal ischaemia-reperfusion injury. Insulin activates the Akt enzyme that plays a role in cellular metabolism, sugar uptake, storage and cellular survive. According to the molecular explanation, the pathways activated by postconditioning, the activation of PI3K, Akt, MEK and ERK signaling pathways that

help to survive and the production of anti-apoptotic enzymes (RISK) lead to counteract the ischemic injury. The PI3-kinase inhibitor, the Wortmannin, destroys the infarction-decreasing potential of the postconditioning. Thus blocking this pathway is our aim to understand the ischaemic postconditioning better in the renal ischaemia-reperfusion.

In this research program we aim to examine the effects of ischaemic postconditioning after 45 minutes of renal ischemia and 120 minutes of reperfusion. In the group of rats with ischaemic postconditioning we used 4 cycles of 15-second-reperfusion and 15-second-reocclusion in the beginning phase of reperfusion. We described the ischaemia-reperfusion damage with the levels of TNF-α cytokine in serum and peripheral tissue. The amount of the ischaemia-reperfusion damage in renal tissue was shown by HE and TNF-α immunhistochemistry with light microscopic technics.

3. Materials and methods

56 male rats of the Wistar strain (bodymass: 300±50g) were used in the studies. In our experiment streptozolocin (STZ: 65 mg/kg ip, Sigma Chemical Co., Budapest) was dissolved in 0,1M citrate buffer (pH 4,5) and this was injected intraperitoneal to induce type 1 diabetes mellitus. The control group was injected with subcutaneous physiological salt (65mg/kg). The blood glucose was checked by Accu Check Active (Roche, Hungary) blood-sugar meter from the blood in tail, it was measured regularly. After 5-7 days the animals were selected into the experimental diabetic group in which the glucose-concentration was equal or exceeded the value of 17mmol/L.

The animals were anaesthetized with an intraperitoneal injection of Calypsol (500 mg ketamine-chlorate pro ampul) and Seduxen (10 mg diazepam pro ampul). The proportion is 1:1 (0.2 ml/100 bwg). The animals lied on the back and were fixed at the legs onto the operation-table. Midline laparotomy was applied and the mesenterial root was mobilised, we checked the similar size of the kindeys, and the bilateral renal arteries and veins were isolated, an atraumatic microvascular clamp was placed on the renal vascular pedicle for 45 minutes to enclose them from the vascular system. We sorted the animals in different groups (n=7). We took sample after the reperfusion. After the 2 hours long reperfusion period, we exsanguinated the animals; blood and serum were used for measuring the following datas. For histological examination the left kidney was fixed in formalin.

Laboratory examination

Glucose, cholesterol, triglyceride, ROS-production, determination of TNF-α cytokine. The examination was conducted as mentioned before.

Histological examination

The left kidney was fixed in 10% neutral-buffered formalin, dewatered with alcohol and paraffin-embedded. 5 μm thick slices were cut according to the standard procedure. A part of the sections were examined by haematoxylin-eosin (HE). We examined all layers of the kidney and the number of hyalincylinders. Jablonski-score and Banff-score were used for grading the tubuli. Monoclonal anti-mouse TNF-α agent was used at the

immunhistochemistry, this has a cross reaction with other rodents, so with the TNF-α in rats as well. This monoclonal antibody paints the cytoplasma of the TNF-α producing cells. The laboratory and analizing procedures are detailed in the Page 6.

4. Results

Laboratory tests

In the cases of streptozotocin-induced diabetes mellitus fasting blood glucose level was significantly greater than at the normal diet group. At STZ-diabetes serum TNF-α levels were significantly lower than in the control group. There was no difference between the groups in the serum creatinine levels before surgery. In each of the four groups increased the concentration of creatinine after surgery. The concentration of serumcreatinin was lower in the control postconditioned and in the with insulin postconditioned groups compared to the IR group. In the Wortmannin-inhibited, ischaemic postconditioned group was the concentration of creatinine higher than in the I/b. and I/c. groups. In STZ diabetes mellitus was the concentration of serum creatinine preoperative higher than in the healthy group. Creatinine concentrations were significantly higher in STZ-induced diabetes mellitus with ischemic postconditioning and Wortmannin-treated group. Ischaemic postconditioning reduced the TNF-α concentrations compared to the healthy control IR group. In the after 45 minutes of ischaemia insulin-treated group and in the group with Wortmannin-inhibited ischaemic postconditioned group significantly increased the concentration at the end of reperfusion. At STZ-induced diabetes mellitus the ischaemic postconditioning did not significantly reduce the concentration of TNF-α compared to the IR group. In the Wortmannin-inhibited ischaemic postconditioned group the concentration increased significantly at the end of reperfusion.

Morphological findings:

In HE-sections of healthy, control group there was no difference found between the groups. Jablonksi 0-2 and Banff Grad I phases were seen. Significant differences were seen in the sections from the STZ diabetic IR group where Grade II severity of multifocal tubular atrophy was observed in the corticomedullar transitions. The amount of TNF-α in the papilla epithelium was quantified and statistically evaluated. From the normal diet-treated groups in the postconditioned group was a significantly lower value detected than in the ischeamia-reperfusion group (I/a.). The concentrations of TNF-α were significantly higher in the insulin-postconditioned group than in the ischaemic postconditioned group (I/b.). In the STZ-induced diabetes mellitus group higher values were detected in all groups than in the control group. Significant differences within the groups were not observed.

5. Discussion

We investigated the protective effects of ischaemic and pharmacological postconditioning in healthy and STZ-induced diabetes mellitus cases. There was no difference in preoperative serum creatinine levels between the groups. In the Wortmannin-inhibited, ischaemic postconditioned group was the concentration of creatinine higher compared to the other groups. The creatinine concentration was significantly higher in the cases with STZ diabetes mellitus in ischaemic postcondicioned, Wortmannin-treated group than in the cases of the insulin-treated group.

The TNF-α is an important cytokine in IR injury. The extent of ischaemia-reperfusion injury was characterized be the levels of TNF-α produced by the leukocytes and endothelium. Our experiments have shown that in healthy, normal diet animals the postconditioning significantly reduced the serum levels of TNF-α. In the after 45 minutes of ischaemia insulin-treated group and in the group with Wortmannin-inhibited ischaemic postconditioned group significantly increased the concentration at the end of reperfusion. At the STZ-induced diabetes mellitus occurred ischaemia-reperfusion injury was greater than in the control group, but it did not affect the ischaemic postconditioning, neither the usage of insulin in the beginning of reperfusion. The pharmacological postconditioning insulin did not reduce the serum-TNF-α levels in either groups.

In HE-sections of healthy, control group there was no difference found between the groups. Jablonksi 0-2 and Banff Grad I phases were seen. It is assumed that the 2 hours reperfusion period is short, it is still even a phase without microscopic changes (HE).

Significant differences were seen in the sections from the STZ diabetic IR group where Grade II severity of multifocal tubular atrophy was observed in the corticomedullar transitions. As it is already written in the first chapter, after repeating the experiment could be established again, that TNF-α was detectable in both groups in the renal cortex and medulla. The amount of TNF-α in the papilla epithelium was quantified and statistically evaluated. In the postconditioned group significantly lower values were detected than in the IR group. In the group with STZ-induced diabetes mellitus the postconditioning did not alter the amount of TNF-α level.

VI. CONCLUSIONS

The prevention, and treatment of ischaemia-reperfusion-induced injury is an important pillar to the success of organ transplantation. We believe that with ischemic postconditioning the acute reperfusion injury can be decreased. In addition, it also has a long-term effect. According to Zhao et al, the achieved reduction of infarct-area after myocardial ischemia can be maintained for 24 hours. This observation is a further evidence for the assumption that the influence of the early events can influence the future events. This phenomenon was originally called ischaemic postconditioning, but it might be better to leave the adjective as it is not clear yet, if it is really the reocclusion or the short episodes of reperfusion are responsible for the protective effects.

The postconditioning drew the attention of the scientific world to the reperfusion as a biologically active and therapeutically important target. Furthermore, postconditioning can be associated with rapidly activating survival kinases, thereby supporting their role in determining the extent of post-ischaemic injury.

The postconditioning is tested in more laboratories in various models, such as small and large animals, in vivo, ex vivo in perfused organs and in cell cultures. The final challenge is, how can the theory of postconditioning theory and experimental experience be implented in our daily clinical practice.

Postconditioning might have a beneficial effect in the level and development of systemic inflammatory response. After 45 minutes of renal ischeamia tha postconditioning reduces the serum concentration of TNF-α and TNF-α signs in kidney papilla in healthy animals. In the present study the initial processes after revascularisation, so the local inflammation both in kidney and in liver, could be inhibited. In the postconditioned group, which was inhibited with Wortmannin, did not decrease the ischaemia-reperfusion injury. It can be concluded that the PI3K-Akt pathway plays an important role in the signal transduction mechanism of ischaemic postconditioning.

In summary, with our research it could have been demonstrated that ischaemic postconditioning can attenuate ischaemia-reperfusion injury at the cases of vessel-operations with kidney-exclusion.

Ischaemic postconditioning was not beneficial to the renal functions in our experiment. The postconditioning did not significantly reduce the levels of serum carbamid; pertaining to serum creatinin levels, the same degree of renal failure was established in each group, so the short-term clinical outcome was not affected. Our results may be related to the duration of reperfusion brevity.

Our further observation was, that hypercholesterolemia alone can enhance the TNF-α production in the renal tubular epithelium and in vascular smooth muscle cells. The

postconditioning reduces the TNF-α levels in the healthy organs, though this effect does not occur in hypercholesterolemic organs.

Peripheral insulin resistance was established during aging in metabolically healthy 5-6-months old Wistar rats. During oral glucose testing not only insulin release can be observed, but simultaneously the serum levels of TNF-α also increase and this plays a role in insulin resistance.

Activity of antioxydant system in liver changes in targeted renal ischemia-reperfusion, and postconditioning has positive effects on that.

In streptozotocin-induced diabetes mellitus the amount of ischemia-reperfusion injury occurred more than in the control group, but it was not affected by ischemic postconditioning, neither the usage of insulin in the beginning of reperfusion. The pharmacological postconditioning with insulin did not reduce the acute inflammatory response. In STZ diabetes mellitus significant differences were detected on the sections in the ischaemia-reperfusion group, multifocal tubular atrophy of Banff Grade II severity was observed there in the corticomedullar transition.

Based on our research, we can say that our experiments examined mechanisms that induce endogenous adaptation - the ischaemic postconditioning - can significantly reduce the oxidative stress, occured during ischaemia-reperfusion, and the inflammatory reaction as well in a healthy body.

The clinical importance of this can be realized after all surgery, where prior ischemic or hypoxic areas are connected into the circulation (vascular and cardiac surgery, traumatic limb injuries, transplantation).

In terms of efficiency, speed and simplicity ischaemic postconditioning seems to be a suitable opportunity after surgery to reduce reperfusion injury.

It makes difficult to reach a high efficiency of the method, if it is used in a healthy or a sick body, from which it is worth of mentioning the large number of metabolic disturbances and their occurrences (hypercholesterolemia, diabetes mellitus).

VII. SUMMARY AND NEW RESULTS

We investigated for the first time the ischaemic and pharmacological postconditioning at renal ischaemia-reperfusion in rat models with metabolic disorders.

I a. Hypercholesterolemia and hypertriglyceridemia was developed after 8 weeks in male rats with a diet that contains 1,5% cholesterol.

b. After the occlusion of both renal hili, after 45 minutes of ischaemia and 120 minutes before reperfusion ischaemic postconfitioning reduce the amount of oxygen free radicals in whole-blood, like the levels of serum peroxid and TNF-α, so that the acute inflammatory reaction.

c. Hypercholesterolemia itself is capable to induce the endogenous protection. At the hypercholesterolemic rats the ischemia-reperfusion injury occurs in a lower amount, it also can alone increase the TNF-α production in the renal tubular epithelium and in the vascular smooth muscle cells.

d. It is assumed that NFKB and MAPK pathways can be induced by the hypercholesterolemia already before the TNF-α receptor-associated pathway. This way it is possible that because of the early response the ischaemia can not induce a significant inflammatory response.

e. Ischaemic postconditioning was not beneficial to the renal functions in our experiment. The postconditioning did not significantly reduce the levels of serum carbamid; pertaining to serum creatinin levels, the same degree of renal failure was established in each group, so the short-term clinical outcome (2 hours) was not affected. Our results may be related to the duration of reperfusion brevity.

II. a. Type 2 diabetes mellitus was developed with a diet that contains 2% cholesterol, 0,5% bile-acids and 20% sunflower oil.

b. During the oral glucose test it could be demonstrated in rat models that not only insulin release can be observed, but simultaneously, the levels of serum- TNF-α increase too, playing so an important role in the development of insulin resistance. Peripheral insulin resistance was established during aging in metabolically healthy Wistar rats.

c. Ischaemic postconditioning reduces the ischaemia-reperfusion injuries only in level of liver MDA following the 90 minutes of ischaemia and 240 minutes of reperfusion.

d. The SOD and glutathione concentration significantly decreased in T2DM after renal ischaemia-reperfusion, indicating the usage of them because of increased ROS-production.

e. The concentration of malondialdehyde was significantly increased in the hepatic tissue in group T2DM compared to the healthy control group.

III. a. Type 1 diabetes mellitus was developed with Streptozotocin. Ischaemic postconditioning reduced the TNF-α concentrations in serum and the TNF-α positivity in renal papilla after 45 minutes of ischemia in healthy rats.

b. The concentration increased significantly at the end of reperfusion in the insulin postconditioned group and in the ischemic postconditioned group, that was inhibited with Wortmannin. The inhibitor of PI3-kinase, the Wortmannin, reduced significantly the beneficial effects of ischaemic postconditioning in the control group. It can be concluded that the PI3K-Akt pathway plays an important role in the signal transduction mechanism of ischaemic postconditioning.

c. In light-microscopic HE-sections of healthy, control group there was no difference found between the groups. Jablonksi 0-2 and Banff Grad I phases were seen. It is assumed that the 2 hours reperfusion period is short, when kidney was removed, it is still even a phase without microscopic changes due to the shortness of the ischaemia-reperfusion period.

d. In the cases of the streptozotocin-induced diabetes mellitus significant differences were seen in the sections from the ischaemia-reperfusion group where Grade II severity of multifocal tubular atrophy was observed in the corticomedullar transitions.

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