The CypD regulates the sepsis and the viability of tumour cells

PhD thesis

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Abbreviations

Akt/PKB;protein kinase B; **ANT**; adenine nucleotide translocase; **Bak**;BCL2-antagonist/killer; **Bax**;BCL2-associated X protein**;COX-2**; cyclooxygenase-2; **CsA**; cyclosporine A; **CypD**; cyclophilin D; **ECSIT**;evolutionarily conservedsignalling intermediate in Toll pathways; **ERK**;extracellular signal-regulated kinase; **FOXO**;forkhead box gene group; **IFN-γ**;interferon-γ; **IL**; interleukin; **iNOS**; inducible NO-synthase; **JNK**; c-Jun N terminal kinase; **LBP**; lipopolysaccharide binding protein; **LPS**; lipopolysaccharide; **MAPK**; mitogen-activated; protein kinase; **MKP**;MAPkinase phosphatase; **MD-2**; myeloid differentiationprotein-2; **MyD88**;myeloid differentiation primary response gene 88; **NF-κB**; nuclear transcription factor-kappa B; **ROS**; reactive oxygen species; **SOD**; superoxide dismutase; **TLR**;**TBK1**; TRIF/TANK-binding kinase 1; **TLR**; Toll-like receptor; **TRIF**; TIR-domain-containing adapter-inducing interferon-β; **TNF-α**;tumour necrosis factor-α; **VDAC**, voltage dependent anion channel.

Introduction

Sepsis

Sepsis is a two-phase systemic inflammation response reaction to various microorganisms (bacteria, fungi, viruses). The first hyper-inflammatory stage of sepsis is characterized by strong systemic inflammation and excessive immune function, while the second hypo-inflammatory phase, which compensates the first phase, is featured by significantly decreased immune function and inhibited inflammation processes. Despite the long decades of research, sepsis is still one of the most common causes of death. Sepsis is actually treated with antibiotics during the intensive care treatment, but even so, it has a mortality rate of 10% in the young, 38% in the older than 85 years old population in the United States. The death of these patients due to the adequate hospital intervention is occurred mostly not in the hyper-inflammatory phase but in the hypo-inflammatory period. They die in the latest phase due to the secondary infections because of the greatly reduced immune reaction and the dissatisfactory function of the internal organs,which are formed in the hyper-inflammatory stage. However, the antibiotic therapy can’t provide protection for a long time against sepsis because of the appearance of multidrug-resistant bacteria.

LPS signalling

Sepsis could be triggered by the LPS of Gram-negative bacteria connecting to the TLR4 receptors of several immune cells and organs. The LPS can classically activate the monocytes in the blood and the macrophages in the tissues. The dissolved LPS first binds to LPS binding protein (LBP), then this complex joins to the CD14 protein and finally to the TLR4/MD2 receptor complex on the surface of the cell. After the activation of TLR4 receptor in the peritoneal macrophages and in the RAW264.7 macrophage-like cell lines the MAP kinases (ERK1/2, JNK1/2, p38) and the NF-κB (p50/p65) transcription factor are activated by phosphorylation through both the MyD88-dependent and MyD88-independent signalling pathways. However, the NF-κB protein activation depends on the acetylation, too.The activated MAP kinases phosphorylates different transcription factors, which together with the phosphorylated NF-κB (p50/p65) transcription factors trigger the expression of inflammation genes (IL-1β, IL-6, IL-12, TNF-α, iNOS, COX-2).After the LPS treatment, the protein level of the MKP1 increases, which dephosphorylates the MAP kinases. MKP1 dephosphorylates theJNK1/2 and p38more than the ERK1/2 protein. The MKP1dephosphorylatesthe JNK1/2 and P38kinases more than the ERK1/2 proteins.After the (100/200 ng/ml) LPS-treatment, theincrease of the MKP1 protein level appears later than the activation of MAP kinases in the(RAW264.7) macrophages. The activation of MAP kinases reaches the maximum level 15 minutes after the LPS stimuli, while the protein level of MKP1 reaches the highest levelonly 60 minutes after the LPS treament. Thereafter, the MAP kinases phosphorylation drops almost to the control level 120 minutes after the LPS treatment, which isfollowed a little bit laterby the decreaseof the protein level of MKP1.Not only the MKP1decreases the activation of MAP kinases but the Akt1 too, which is activated after the LPS stimuli. Furthermore, under the LPS treatment the Akt protein is activated through the PI3K/Akt and TRIF/TBK1 pathways and inhibits the inflammation by decreasing the phosphorylation of the MAP kinases and NF-kB (p50/p65). Besides these, the activation of Akt1 further increases after the (100ng/ml or 200 ng/ml) LPS treatmentin the 120th minute contrary to the decreasingactivation of MAP kinases andprotein level of MKP1. The late increase of Akt1 activation is indispensable for the phenotypic and functional switch of macrophages and need to appear M2 type macrophages which secrete anti-inflammatory cytokine (IL-10), and regenerate the tissue by the production of extracellular components like fibronektin and protein (βIG-H3) which bind to the extracellular matrix. Besides, the activated Akt

phosphorylates inactivates the FOXO1 and FOXO-3a transcription factors, which in activated condition reduce the level of oxidative damage or provoke macrophage apoptosis because of the excessive oxidative damage.

The effect of CypD and LPS on the pore openings and mitochondrial ROS production

The CypD protein is encoded by the Ppif gene in the nucleus. The N-terminal target sequence of this protein needs to get into the matrix of mitochondrion, thereafter this sequence falls off. The CypD proteins connect directly to the F_oF_1 ATP synthase dimers, the ANT and the PiC proteins and initiate the pore opening in the mitochondrial inner membrane.The CypD proteins most likely create pores via the peptidil-prolil-cis-trans isomerization of these proteins.Besides, the CypD reduces the synthetic and hydrolytic activity of the F_oF_1 ATP synthase, which inhibits the restoration of the membrane potential and the ATP synthesis after the internal pore openings.However, the CypD depletion or the inhibition of CypD does not only regulate the inner pore opening, which is indicated by swelling of the isolated mitochondrion of liver and nerve cells, but it is also responsible for triggering the outer pores opening. It is indicated with the release of pro-apoptotic factors from the inter-membrane space to the cytosol. We can meet this phenomenon in the oxidative stress induced nerve tissue, in the LPS-treated liver and in the myocardium after ischemia-reperfusion. The outer membrane pores are formed by either Bax and/or Bak homo- or hetero-oligomers, or Bak or Bax and VDAC hetero-oligomers. In the course of the LPS-induced signal transduction the extent of the phosphorylation of CypD's amino acids probably determines the pore forming in the inner and outer membranes. The Akt2 and GSK-3B phosphorylate the CypD protein which inhibits the inner pores formation of PiC, ANT proteins and F_oF_1 ATP synthase dimers. Besides, the deacetylation of CypD protein inhibits the opening of mPTP by the Sirt3 (Sirutin3), too. Furthermore, the CypD is required to directly connect to PPARalpha to open the pores. After the CypD triggers mitochondrial inner pores opening, the fluid and the ions flow from the cytosol and intracristal space to the matrix, which reduces the membrane potential. The inner pore openings and the membrane potential reduction induce the removal of the electrons mainly from the NADH dehydrogenase and the ROS production of mitochondria. Besides, the LPS activates the ECSIT proteins by ubiquitination, which also triggers the superoxide radical production of NADH dehydrogenase. According to the literature, the mitochondrial superoxide radical production is the main source of the cellular reactive oxygen species. We can also read in the literature that the oxidative stress is

essential for NF-kB (p50/p65) transcription factor activation, which contributes to the ERK1/2 and p38 activation and decreases the JNK1/2 activation in the macrophages. In addition, after the FOXO-3a and the FOXO1 transcription factors are activated by phosphorylation, they reduce the harmful effects of oxidative stress by increasing the transcription of antioxidant enzymes after the LPS treatment. However, if the oxidative stress is excessive, these transcriptions factors evoke apoptosis.

The inhibition of CypD with cyclosporineA antibiotic and polyphenols

CsA is an antibiotic and an inhibitor of CypD, which connects to the CypD protein and prevents the CypD connection to the internal pore forming proteins. Therefore, in the case of intact mitochondria the CsA inhibits the internal pore opening and the swelling of mitochondria. At the same time, the CsA inhibits the ROS production of neurons in the Alzheimer's disease and prevents the opening of outer mitochondrial pores and apoptosis in the liver in course of sepsis and in the myocytes in course of ischemia-reperfusion. However, the CsA inhibits the expression of MKP-1 protein through the calcineurin pathway and thus enhances the activation of MAP kinases and inflammation. Moreover, the CsA even inhibits the cyclophilin A, B and C proteins. The inhibition of cyclophilin A exerts immunsuppressant effects. Besides, CsA prevents the pro-inflammatory cytokine secretionby inhibiting the cyclophilin A and B. Thus it cannot be regarded as a specific inhibitor of CypD.

We can read in the literature that resveratrol and ferulic acid belong to the polyphenols and inhibit the LPS-induced inflammation in macrophages via the inhibition of the activation of MAP kinases and the NF-kB transcription factor. Besides, the resveratrol inhibits the membrane depolarization, the ATP synthesis and the internal pore opening of the mitochondrion. The resveratrol can increase the GSK-3β (Ser9) phosphorylation, which moves into the mitochondrion to connect to the CypD and this way it inhibits the inner pore opening.Probably because of their same functional groups, which are in the same spatial position, resveratrol and ferulic acid inhibit the LPS-induced inflammation in the same way. Thus, it is most likely that the ferulaldehyde which differs from ferulic acid in only one amino group, will also affect the inner pore opening of the mitochondria.

Poly (ADP-ribose) polymerase (PARP) enzyme has 17 isoforms. PARP enzymes can be activated by several stimuli. PARP1 and PARP2 poly-ADP-ribosylate the single-stranded DNA (ssDNA) breaks and this way indicate the location of the breaks for the DNA repair system.

PARP1 can poly-ADP-ribosylate in itself and thus connect to the DNA-Methyltransferase and inhibits the imprinting, too. The activation of PARP-1 enzyme can be triggered by single-stranded DNA breaks appearing in increased oxidative stress and also LPS stimulus. PARP1 activation has a main role in the LPS-induced inflammation processes. It is proved by two articles, which reported that 90% of PARP1-/- mice survived the lethal LPS (40 mg/body weight kg) treatment and the lack of PARP1 inhibited the (1 μg/ml) LPS-induced NF-kB activation and the expression of NF-kB-dependent genes in peritoneal macrophages. In the latter casePARP1 as the coactivator of NF-kB enhances the expression of inflammatory genes. In this way PARP1 poly-ADP-ribosylates the histone proteins and therefore decondensates the chromosomes and lets the expression of inflammatory genes. The activated NF-kB, Egr1 and several transcription factors are able to generate the gene expression of PARP1, too. It can be stated from PARP1, that the lower activation of this protein can enhance inflammation and the excessive activation triggers the death of cells. In the latter case, the activated PARP1 evokes the opening of mPTP. Results showed that the PARP1 inhibitors through the PI3K/Akt pathway prevent the mitochondrial ROS production by the enhancement of the phosphorylation of Akt1 (Ser473) and GSK-3β (Ser9). The inhibition of ROS production is indicated by the depolarization induced by the mPTP opening. The p-GSK-3β (Ser9) inhibits the opening of mPTP, probably through the phosphorylation of CypD protein. At the same time, the PAR, which is formed during the activation of PARP1, elicits the pore opening in the outer mitochondrial membrane, thus the pro-apoptotic factors release from the inter-membrane space to the cytosol and the factors induce cell death. PARP1 excessive activation can contribute to cell death by triggering the ATP and NAD^+ store depletion because of PARP1 use NAD^+ for PAR production, and NAD^+ is required in several ATP producing biochemical processes (glycolysis, breakdown of amino acids, β-oxidation). On the other hand, PARP1enzyme can achieve the reduction of the ATP level by inhibiting thehexokinase enzyme as well, which takes part in the glycolysis.Thus, PARP1 activation triggers apoptotic or necrotic cell death by the depletion of the ATP store due to the mPTP opening and decreased hexokinase reaction, ATP synthesis and NAD^+ store.

Aims of study

1. At first we wanted to know whether the CypD induces the inner pores opening and therefore the depolarization of mitochondria in the wild type peritoneal macrophage cells after

the LPS stimulus. Besides, we aimed to determine the extent of the inhibitory effect of CypD deficiency on these phenomena after the LPS stimulus, too.

2. We wanted to know whether the mPTP opening, which is induced by activated CypD,or theubiquitinatedECSIT protein triggers mainly the mitochondrial ROS production in the LPS-treatedmacrophages. Furthermore, we aimed to investigate what effect the mitochondrial ROS production has on the whole cell ROS production.

3. Our aim was to examine whether the lack of CypD decreases the NF-κB activation and the NF-κB-induced genes (TNF-α, iNOS) expression by the inhibition of ROS production in the LPS-treated peritoneal macrophages. In addition, we wanted to know whether the inhibition of oxidative stress by CypD deficiency increases the inactivation of FOXO1 and FOXO-3a transcription factors, which protect the cells against the oxidative stress. Besides, we aimed to examine whether Akt1 protein ensures the inactivation of these transcription factors.

4. We wanted to know whether the ferulaldehyde, a degradation product of ferulic acid, inhibits the mitochondrial depolarization which shows mPTP opening after the LPS treatment in secondary RAW264.7 macrophage cells. In addition, we aimed to know whether the ferulaldehyde inhibits the total cell ROS production by own free radical scavenging ability or inhibition of the mitochondrial mPTP opening in the LPS-treated cells.

5. We wanted to bring to the light whether the ferulaldehyde reduces the activation of MAP kinases, Akt1 and NF-κB mainly by the own free radical scavenging ability or it is its own effect on signal transduction. Besides, we aimed to know whether the ferulaldehyde regulates the phosphorylation of CypD by the phosphorylation of Akt1 and GSK-3β and therefore triggers theopening ofthe mPTP after the LPS treatment.

6. We aimed to study whether the IK11 could trigger mitochondria depolarization which indicates the mPTP opening, ROS production, apoptosis and necrosis of HepG2 cancer liver cell lines. At the same time, we also wanted to know whetherPARP1 activation contributes to the cell death by the mPTP opening after IK11 treatment. Besides, we wanted to know whether PARP1triggers the mPTP opening and the cell death by changing the signal transduction pathways.

Materials and methods

Reagents

All chemicals and cell culture media when not indicated otherwise, Escherichia coli LPS (0127:B8), protease and phosphatase inhibitor cocktail, Calcein-acetomethoxy (AM) derivate, A23187, dihydrorhodamine 123 and peroxidase conjugated anti-mouse IgG secondary antibody were from Sigma-Aldrich Hungary (Budapest, Hungary). Horseradish peroxidase conjugated anti-rabbit IgG secondary, anti-phospho-forkhead box O (FOXO)-1 and FOXO-3a primary antibodies was from BIO-RAD Hungary (Budapest, Hungary). Anti-phospho-Akt (Ser 473), anti-phospho-NF-κB p65 (Ser536), anti-phospho-44/42-MAPK (Thr202/Tyr204), anti-phospho-p38-MAPK (Thr180/Tyr182), anti-phospho-SAPK/JNK (Thr183/Tyr185) and anti-NF-κB p65 primary antibodies were from Cell Signalling Technology (Danvers, MA, USA). Anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody was used in the sample of HepG2 cell was from R&D System. The MKP-1 (C-19) primary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and anti-histone H1 primary antibodies were from Millipore Merck Ltd. (Budapest, Hungary) and Abcam (Cambridge, UK), respectively. Co (II) chloride-6-hydrate was from Reanal (Budapest, Hungary). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein-diacetate (C-400) were from Molecular Probes (distributed by Csertex Ltd, Budapest, Hungary). The used kinase pathway inhibitors, namely Akt pathway inhibitors (LY294002, Akt inhibitor IV.) and JNK inhibitor (SP600125) were purchased from Calbiochem. All other substances including trans-resveratrol and PJ34 were from Sigma-Aldrich. IK11, was synthesized by us and was dissolved in dimethyl-sulphoxide (DMSO) at 1000 times of the final concentration used. The HO3089 and the L2286 PARP inhibitors as well as the ferulaldehyde were kind gifts of Professor Kalman Hideg (Department of Organic and Pharmacological Chemistry, University of Pecs Medical School, Pecs, Hungary).

Mice

The male CypD $^{-/-}$ and mice with C57BL/6 background were supplied by Prof. László Tretter (Semmelweis University, Budapest, Hungary). The mice were mice were kept under standardized conditions according to the Guide for the Care and Use of Laboratory Animals published by the USNIH. Tap water and mouse unpurified diet consisting of maize, barley, wheat, milk powder,

baker's yeast, lime and soya (Szindbad Ltd, Gödöllő, Hungary) were provided ad libitum. Animal experiments were covered by a licence issued by the Regional Food Safety and Animal Care Directorate, and all experimental procedures were approved by the Animal Research Review Committee of the University of Pecs, Medical School.

Cell cultures

Primary resident peritoneal macrophages were isolated according to the protocol of Zhang et al. from $CypD^{-/-}$ and wild-type 16-20 weeks old mice of 22–35 g body mass. The peritoneal macrophages and the RAW264.7 cells were seeded to 6- or 96-well plates in endotoxin tested Dulbecco's modified Eagle's medium containing 0.1% penicillin-streptomycin, 10% fetal calf serum and 8 mM L-glutamine. Experiments were performed on 80% confluent plates. HepG2 human hepatocellular carcinoma cells obtained from European Collection of Cell Cultures were cultured in 5% CO_2 at 37°C in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. Cells were seeded at a starting density of 2×10^4 cells/well in a 96-well plate for viability and ROS production assays, or of 2×10^6 cells/well in a 6-well plate for immunoblotting and determination of cell morphology.

Immunoblotting

Cells in 6-well plates were treated with 1μg/ml LPS for 90 min, washed once with phosphate buffered saline (PBS), and lysed by sonication in 100 μl solution containing 50 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 150 mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM ethyleneglycoltetraacetic acid (EGTA), 0.1% Polyoxyethylene (20) sorbitan monolaurate (Tween-20), 10% glycerol, 0.1 mM Na_3VO_4, 10 mM 1,4-dithiothreitol (DTT), and protease and phosphatase inhibitor cocktail. To the cell lysates, 2x Laemmli sample buffer was added, the samples were boiled for 5 min and centifuged at 17000 g for 5 min.

RAW264, 7 cells ($2x10^6$)/well were seeded into a six-well plate and incubated for one night. Later the cells treated or not with 100 ng/ml LPS, with or without 50 μM ferulaldehyde for 10 or 30 min. After the treatment the cells were harvested in ice-cold lysis buffer containing 0.5 mM sodium metavanadate, 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor mixture in phosphate-buffered saline (PBS). The HepG2 cells were treated with 1000 times diluted DMSO as vehicle control (CTRL) or 10 μM IK11 and/or 10 μM of the PARP inhibitor

PJ34 (applied 1 h before the IK11 treatment) in different combinations as indicated for 6 h. After the treatment the cells were harvested in ice-cold lysis buffer containing 0.5 mM sodium metavanadate, 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor mixture in phosphate-buffered saline (PBS). After the lysis of HepG2 and RAW cells the proteins were precipitated by trichloroacetic acid, washed three times with −20°C acetone. To the sediment 1x Laemmli sample buffer was added.

In the case of HepG2, RAW264,7 and intraperitoneal macrophages 20 μg proteins were separated on 10% sodium dodecylsulphate gels, and blotted to nitrocellulose membranes. Membranes were blocked in non-fat milk, were exposed overnight at 4˚C or 2 hours at h at room temperature to primary or secondary antibodies at a dilution of 1:1000 or 1:5000, respectively. Bands were visualised by enhanced chemiluminescence (AP Hungary Ltd, Budapest, Hungary). Pixel densities were normalised to that of the loading control. All immunoblotting experiments were repeated at least three times.

The measurement the activation of NF-κB with NF-κB biding oligonucleotide

Preparation of nuclear protein extracts

The nuclear extracts were prepared as described previously (Tang 2007). CypD-/- and wild-type peritoneal macrophages were harvested and suspended in hypotonic buffer A (10mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), pH 7.6, 10mM KCl, 1mM dithiothreitol (DTT), 0.1mM EDTA, and 0.5mM phenylmethylsulfonyl fluoride) for 10min on ice and vortexed for 10s. Nuclei were pelleted by centrifugation at 12000g for 20 s. The supernatants containing cytosolic proteins were collected. Nuclear pellet was suspended in buffer C (20mM HEPES, pH 7.6, 1mM EDTA, 1mM DTT, 0.5mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4M NaCl) for 30 min on ice. Nuclear protein containing supernatants were collected by centrifugation at 12000g for 20 min and stored at −70°C.

DNA affinity protein binding assay

After the indicated treatment, peritoneal macrophages were harvested in buffer A, chilled on ice for 10 min and centrifuged at 12000g for 20 s. Pellets were suspended in 5 times volume of buffer C and sonicated. A 200μg aliquot of nuclear suspension was incubated with 2μg of biotinylated double-stranded oligonucleotyde corresponding to the murine consensus NF-κB binding DNA sequence (Biotin-CCTTGAAGGGATTTCCCTCC, Invitrogen) for 30 min on a

4ºC shakerbath. Then, 30µl streptavidin coated magnetic micro particles (Sigma-Aldrich) were added, and incubation was continued for an additional 30 min. Beads were pulled down, washed 3 times with ice-cold PBS, and eluted in 25µl mercaptoethanol-free Laemmli sample buffer by a 5 min boiling. Eluted samples were subjected to immunoblot analysis. All experiments were repeated three times.

Determination of NF-κB activity by using NF-κB luciferase assay

RAW 264.7 macrophages were transiently cotransfected with either NF-κB luciferase (NF-κB) or control (TA-Luc) (Panomics, Santa Clara, CA, USA), and SV-β-galactosidase (pSV-β-gal) (Promega Corporation, Madison, WI, USA) plasmids by using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. 24 h after the transfection cells were treated as indicated, and another 24 h later cell lysates were collected. Cellular proteins were assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA, Luciferase Assay System Technical Bulletin TB281). The ratio of luciferase to β-galactosidase activity served to normalize the luciferase activity to correct for any differences in transfection efficiencies.

Tumour necrosis factor (TNF) α measurement

$CypD^{-/-}$ and wild-type peritoneal macrophages in 6-well plates were treated or not by 1µg/ml LPS for 90 min, then TNFα content in the culture supernatants was measured by using the Quantikine M TNFα enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK) according to the manufacturer’s protocol. These experiments were repeated three times.

Silencing of PARP1 by siRNA technique in HepG2 cells

HepG2 cells were transiently transfected with siRNA designed for PARP1 suppression by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA) in Opti-MEM I Reduced Serum Medium (Invitrogen) using Lipofectamine 2000. For an effective suppression of PARP, the transfection step was repeated twice with a 48-h interval between the transfections, and the experiments on the cells were performed 40 h after the third transfection.

HepG2 cell viability assay

Cells were seeded at a starting density of 2×10^4 cells/well in a 96-well plate and incubated for one night. Later the HepG2 cells were treated with 1000 times diluted DMSO as vehicle control (CTRL) or 10 μM IK11 (solved in 1000 times diluted DMSO) and/or 2 mM NAC (ROS scavenger), 50 μM resveratrol, 10 μM PJ34 PARP inhibitor, 10 μM of the JNK inhibitor SP-600125, 32.54 μM of the Akt pathway inhibitor LY294002, 5 μM of the specific Akt inhibitor Akt-inh. IV. or 50 μM of the ROS scavenger in different combinations for 24 hours. The PJ34 PARP inhibitor was added to the cells one hour before the IKK11 treatment. SiRNA transfected HepG2 cells to silencing of PARP were treated with 10 μM IK11 (solved in 1000 times diluted DMSO) or 1000 times diluted DMSO for 24 hours. After the treatment, medium was replaced to a fresh one containing 0.5% MTT. Incubation was continued for an additional 3 h, and the reduction of MTT to formasan was terminated by adding isopropanol containing 0.4% HCl. The concentration of the water-insoluble formasan dye was proportional to the number of living cells. After dissolving the dye in the acidified isopropanol, the absorption was measured with an Anthos Labtech 2010 plate-reader at 550 nm wavelength. All experiments were run in 6 parallels and repeated three times.

Measurement of nitrite concentration

$CypD^{-/-}$ and wild-type peritoneal macrophages were treated or not by 1 μg/ml LPS for 24 h. The RAW264.7 cells were treated or not by 100 ng/ml LPS and/or 1-100 μM ferulaldehyde or only by 100 μM ferulaldehyde for 24 h. Ferulaldehyde was added 5 minutes before the LPS treatment. Then nitrite production was measured by adding to a 50 μl culture supernatant an equal volume of Griess-reagent (1% sulphanilamide, 0.1% naphthylethylenediamide in 5% phosphoric acid) and measuring light absorption at 550 nm using a Glomax Multi+ multimode reader. These experiments running in at least six parallels were repeated three times.

Determination of mitochondrial ROS production

$CypD^{-/-}$ and wild-type peritoneal macrophages were seeded into 96-well culture plates and incubated overnight. Culturing medium was replaced with a fresh one containing 1 μg/ml LPS for 2, 10 or 22 h, then dihydrorhodamine 123 at a final concentration of 2 μM was added to the medium for an additional 2 h. Fluorescence was measured at 485 nm excitation and 520 nm

emission wavelengths by using a Glomax Multi+ multimode reader. These experiments running in at least six parallels were repeated three times.

Determination of cellular ROS production in RAW264.7, HepG2 and peritoneal macrophage cells

CypD$^{-/-}$ and wild-type peritoneal macrophages were treated or not with 1 μg/ml LPS. The RAW264.7 cells were treated or not with 100 ng/ml LPS and/or 1-100 μM ferulaldehyde or only with 100 μM ferulaldehyde. Ferulaldehyde was added 5 minutes before the LPS treatment. After the LPS treatment the RAW264.7 and peritoneal macrophages were incubated for LPS for 22 h. The HepG2 cells were seeded at a starting density of 2×10^4 cells/well in a 96-well plate and incubated for one night. Later the HepG2 cells were treated with 1000 times diluted DMSO as vehicle control (CTRL) or 0,1-10 μM μM IK11 (solved in 1000 times diluted DMSO) and/or 2 mM NAC (ROS scavenger), 10 μM PJ34 PARP inhibitor, in different combinations for 24 hours. The PJ34 PARP inhibitor was added to the cells one hour before the IKK11 treatment. SiRNA transfected HepG2 cells to silencing of PARP were treated with 10 μM IK11 (solved in 1000 times diluted DMSO) or 1000 times diluted DMSO for 24 hours as well. After the treatments C400 at a final concentration of 2 mg/L was added to the medium of the intraperitoneal macrophages, RAW264,7 and HepG2 cells for an additional 2 h. Fluorescence was measured at 485 nm excitation and 555 nm emission wavelengths by using a Glomax Multi+ (Promega, Mannheim, Germany) multimode reader. These experiments running in at least 6 parallels were repeated three times.

Detecting mitochondrial membrane potential (Δψ)

CypD$^{-/-}$ and wild-type were treated by 1 μg/ml LPS for 90 min. The Raw264.7 cells were treated by 100 ng/ml LPS and 50 μM ferulaldehyde for 5, 10, 30, 60 minutes. The ferulaldehyde was added 5 minutes before the LPS treatment. HepG2 cells were treated or not (CTRL) with 10 μM IK11 for 24 hours. Later the peritoneal and Raw264.7 cells were dyed by 2 μM JC-1 and/or 100 nM tetramethylrhodamine-methyl-ester (TMRM) (Invitrogen), then were immediately analysed by a BD FacsCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were accumulated and reduced by Cellquest software (BD Biosciences, San Jose, CA, USA). JC-1 is accumulated in the mitochondria in a potential-dependent manner indicated by a fluorescence emission shift from red (≈590nm) to green (≈529nm) upon depolarization. Consequently,

mitochondrial depolarization is indicated by decrease in the red/green fluorescence intensity ratio. The TMRM is a potential dependent dye emits increased fluorescent intensity (≈585 nm) at ≈543 nm excitation wavelength when the mitochondria are depolarized. Cells in each category were expressed as percentage of the total number of stained cells counted. These experiments were repeated three times.

RAW264.7 cells were seeded to glass cover slips, treated by 100 ng/ml LPS and/or 50 μM ferulaldehyde, washed twice in ice-cold PBS, and loaded with JC-1 for 15 min at 37°C. When excited at 488 nm, the dye emits green fluorescence when the mitochondria are depolarized, and red for normal ΔΨm. Cells were imaged with a Zeiss Axiovert 25 fluorescent microscope equipped with a ProgRes C12 Plus CCD camera using a 63 x objective and epifluorescent illumination. The same microscopic field was imaged in the red then the green channel, then the images were merged.

RNA extraction and quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR) of MKP1

Total RNA was extracted from RAW 264.7 cells using TRIZol reagent (Sigma-Aldrich Co, Budapest, Hungary), according to manufacturers' protocol. RNA (1 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MuLV RT, RevertAidTM First Strand cDNA Synthesis Kit) (Fermentas, Burlington, Ontario, Canada) for 1 h at 42°C final volume was 20 μl. 1 μl of cDNA was used for real-time PCR. Real-time PCR runs were performed on Corbett Rotor-gene 3000. PCR was conducted over 45 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s three-step thermal cycling preceded by an initial 95°C for 7 s using iQ SYBR Green Supermix Kit (Bio-Rad, Hercules, CA, USA). Statistical analysis of relative expression of the target gene based on Ct values with efficiency correction was made with the Relative Expression Software Tool (Corbett Research, Germantown, MD, USA) normalized to the housekeeping gene GAPDH.

Demonstration of mPT pore opening

CypD-/- and wild-type peritoneal macrophages at a starting density of 10^6 cells per well were seeded onto glass cover slips placed in the wells of a 6-well culture plate and incubated overnight in DMEM medium. Culturing medium was replaced with a fresh one containing 1 μg/ml LPS and the cells were incubated 30 or 60 min. In the last 30 minutes of the LPS treatment calcein-AM

dye was added to the medium of cells. After this the medium was replaced with the Ca2+- and Mg2+-free Hank's balanced salt solution (HBSS), and fresh Ca2+- and Mg2+-free HBSS containing 1 μ calcein-AM 250 nM A23187, 90 μM Co(II)-chloride, 1 g/l glucose and 1 μg/ml LPS was added to the wells. Following 30 min of incubation, the wells were washed once with HBSS, then the cells were incubated in Ca2+- and Mg2+-free HBSS. The cover slips were placed upside down on the top of a small chamber formed by a microscope slide and a press-to-seal silicone isolator. The cells were imaged using a Nikon Eclipse Ti fluorescent microscope equipped with a SPOT RT3 CCD camera, using a 40× objective and bright field or epifluorescent illumination. These experiments were repeated three times.

Image processing

Original fluorescent and bright-field microscopy images in Figure 1 were converted to black and white, cropped to the same size and placed adjacent to each other. For representative blots in Figures 4 and 5, x-ray films were scanned and the images were cropped. No other image manipulation was performed. We used NIH Image J software for image processing and digitalization of immunoblots.

Statistical analysis

Mean ± SEM values were analysed using two-way analysis of variance followed by Tukey's post-hoc test. Differences were considered significant at $p < 0.05$.

Results

1. At first, we investigated whether the LPS induces the mPTP opening in the peritoneal macrophage and the CypD,which according to the literature regulates the opening of mPTP,induces the mitochondrial depolarization by the opening of the inner and outer pores together. Our results made us realize that the LPS (1 μg/ml) triggered the mPTP opening,which was detectable by microscope in the 60^{th} minute in the CypD+/+ macrophages, and it increased further until the 90^{th} minute, too. Besides, significant mitochondrial depolarisation was associated with the mPTPopeningin the CypD+/+ cells 90 minutes after the LPS treatmentwhichwas cancelled with the mPTP opening in theLPS-treated CypD -/- cells.Thus it becomes clear that the CypD provoked the mPTP opening in the CypD-/- cells. Furthermore, the absence of CypD increased the membrane potential by inhibiting the mPTP opening in the control CypD-/- cells,

too. We investigated the mPTP opening in the RAW264.7 cells as well 90 minutes after the LPS (1 μg/ml) treatment. According to our experiments in this cell line we were convinced that the mPTP opening, which was detected by calcein-cobalt dyeing, appeared earlier than the mitochondrial depolarisation which was measured by JC-1 dyeing.Namely, we found in the RAW264.7 macrophage-like cell lines that the mitochondrial membrane is depolarized 5 minutes after the LPS (100ng/ml) treatment and it reached the maximum level 30 minutes later. Thus, we made sure that the CypD is the main regulator of the mitochondrial depolarization by controlling the mPTP opening. Besides this, we saw that the total membrane depolarization, which was detected by JC-1, appeared much earlier than the mPTP opening. It indicates that the depolarization needs less mPTP opening after the LPS treatment.

In the literature we can read that the mPTP opening and the mitochondrial depolarization are associated with the mitochondrial ROS production, and the floating electrons from mitochondrial NADH dehydrogenase are mainly responsible for the mitochondrial ROS production. We found in the literature that the ECSIT protein, which is ubiquitinated after the LPS treatment,can trigger the electrons toget off from the complex I, too. Therefore, we examined, whether the CypD inducesprincipallythe mitochondrial ROS production. Besides, we researched what the effect of mitochondrial ROS production on the whole cell ROS production is.We found in the CypD+/+ cells thatthe mitochondrial ROS production was two times bigger than in the CypD-/- cells. At the same time this difference decreased more and more in the 12^{th} and the 24^{th} hour after the LPS treatment. During this time the increase of the whole cell ROS production 24 hours after the LPS stimulus in the CypD+/+ cells was the same as we measured in the mitochondrion in the 4^{th} hour after the LPS treatment in the same cell type. Thus, the mitochondrial ROS production was not responsible for the whole cell ROS production 24 hours after the LPS treatment in the CypD+/+ cells. In this casean extramitochondrial ROS production appeared, aROS production outside of the mitochondrion, which turned up probably due to the oxidative damageof enzymatic reactions. Besides, the continuously decreasing mitochondrial ROS production emerged likely due to the oxidative damage of an electron transport chain in the CypD+/+ cells after the LPS treatment. In contrast to this, in the CypD deficient cells 4, 12 and 24 hours after the LPS stimulus the mitochondrial ROS production was at the same level, and it was significantly lower than in the LPS-treated mitochondria of wild-type cells. During this time, the increase of the whole cell ROS production 24 hours after the LPS stimulus in the CypD-/- cells was the same as what we had

measured in the mitochondrion in the 4th,12th and 24th hour after the LPS treatment in the same cell type. Therefore, the deficiency of the CypD inhibited the extramitochondrial ROS production, which probably appeared due to the oxidative damage of the enzymatic functions, by reducingthe mitochondrial ROS production.In the CypD-/- cells the ubiquitinated ECSIT protein was likely responsible for the ROS production. It became clear thatthe mPTP opening and not the ECSIT protein is mainly responsible for the mitochondrial ROS production in the LPS-treated CypD+/+ cell. Namely, the ROS production in the CypD+/+ cells was two times bigger than what we saw in the CypD+/+ control cells, and the ROS production in the LPS-treated CypD-/- was just a bit bigger than in the CypD+/+ control cells.

In the literature we can read that the excellent antioxidant, the N-acetil cysteine (NAC) inhibits the NF-κB transcription factor activation in the inflamed lungs by reducing the oxidative stress. Therefore, the total activation of NF-κB needs the mitochondrial ROS production in addition to the signal transduction changes after the LPS treatment. Thus, we examined whether the lack of CypD inhibits the activation and translocation of NF-κB and it inhibits the expression of NF-κB dependent genes (TNF- α, iNOS) as well by reducing the ROS production after the LPS treatment. Beside these, we wanted to know whether the decreased oxidative stress can modify other signal transduction pathways, too. Our results showed that the lack of CypD inhibited the activation of NF-κB, the translocation of NF-κB and the expression of the NF-κB-induced genes in the same extentas the ROS production after the LPS stimulus. According to this data oxidative stress is needed to the total activation of the NF-κB protein. Besides, our results show that due to the reduction of oxidative stress the inactivation of FoxO1 and FoxO-3a transcription factors is increased as well as in the LPS-treated or untreated CypD deficient cells compared to the adequate wild-type cells. The FoxO1 and FoxO-3a transcription factors are inactivated via phosphorylation by Akt1 after the LPS treatment. In the LPS-treated or not treated CypD-/- cells the Akt1 activation and the inactivation of the FoxO1 and FoxO-3a proteins was significantly larger than in the adequate wild-type cells. These FoxO transcription factors are necessary to inhibit the harmful effect of oxidative stress due to the increasing expression of antioxidant enzymes. These factors can initiate the apoptosis as well when oxidative damages of the cells are severe. Thus, the lack of CypD initiated the reduction of oxidative stress and this way decreased the activation of NF-κB, FOXO1 and FOXO3a-transcription factors, while it increased the activation of Akt1. Akt1 is responsible for the inactivation of MAP kinases and NF-

κB protein and it is indispensable for the termination of macrophage classical activation and pro-inflammatory cytokine production.

2. Later our attention was directed to ferulaldehyde, which belongs to the polyphenols as well just like resveratrol andferulic acid. These polyphenols havethe same structural componentand the same functional groups in the same spatial position. In the literature we can find about the resveratrol that it can decrease the damage of myocardium after the ischemia-reperfusion injury by triggering the CypD phosphorylation. In this case the phosphorylated GSK-3β (Ser9) phosphorylatesthe CypD, and consequently inhibits the mPTP opening. Therefore, we were curious whether the ferulaldehyde inhibits the ROS production by decreasing the mPTP opening. We also wanted to know how the decreased oxidative stress modifies the signal transduction in the RAW264.7 macrophage cell after the LPS treament. Our results showed that the ferulaldehyde slightly repolarized the mitochondrial membrane, which indicates the opening of the mPTP. At the same time the ferulaldehyde fully decreased the ROS production of the whole cell in the LPS-treated RAW264.7 cells. Thus, the ferulaldehyde prevented the ROS production of the whole cell mainly due to the antioxidant effect and not because of the reduction of the mPTP opening. Besides, ferulaldehyde inhibited the ROS production of mitochondria to the same extent as the activation of NF-κB and theexpression of a NF-κB-induced gene (iNOS) after the LPS treatment, too. At the same time, the ferulaldehyde inhibited the activation of JNK1/2 and p38 much more than the activation of ERK1/2 in the RAW264.7 cells after the LPS-treatment. This is rather different from the effect of NAC, which is believed to be a good antioxidant. This happened because NAC inhibited the activation of ERK1/2 and p38 and it increased the activation of JNK1/2 in the macrophages after the LPS treament. Thus, it seems that the ferulaldehyde reduced the activation of MAP kinases, but not only through the inhibition of ROS production.This is verified by the results that showed that the ferulaldehyde increased the LPS-induced inhibition of MKP1 phosphatase. The MKP1 is mainly responsible for the dephosphorylation and inactivation of p-JNK1/2 and p-p38 ,but besides this it dephosphorylates the ERk1/2, too. In addition, in the RAW264.7 cells the ferulaldehyde significantly decreased the activation of Akt1, while it totally prevented the phosphorylation of GSK-3β, the target protein of Akt1 after 30 minutes of LPS treatment. Thus GSK-3β protein could not induce the opening of mPTP by directly phophorylating the CypD. In addition, the ferulaldehyde inhibited the activation of Akt1 such as the NAC in the same way. According to these data we can conclude

that the ferulaldehyde inhibited the NF-κB and Akt1 activation by completely removing the ROS due to its own antioxidant properties, while it inhibited the MAP kinase activation by changing the signal transduction after the LPS treatment. To be more precisse, it increased the protein level of MKP1.

3.The subsequent investigations were in another experimental model, in which we examined whether the IK11 induces the cell apoptosis and necrosis through the mPTP opening and how the inhibitors of PARP1 and/or PARP1 can modify it in the HepG2 cancer cells. In the literature we can read that the IK11 can cause PARP1 dependent apoptosis of A431 cancer cell line through the opening of outer mitochondrial pores. We can also find in the literature that in the inflammatory model the inhibition of PARP1 decreases the mitochondrial membrane depolarization, the ROS production and the release of pro-apoptotic factors by inhibiting the PI3K/Akt1/Gsk3β pathway. Therefore, at first we tried to find the IK11 concentration which can induce the apoptosis and necrosis and which can decrease the vilability of cells. To be more precise, this concentration definetely triggers the opening of mPTP and thus induces the appearancethe of depolarization, the mitochondrial and extramitochondrial ROS production, the release of pro-apoptotic factors and maybe it induces the mitochondrial fission, too. According to our examinations, we saw that the IK11 inhibited the cell-division at a concentration of 1 μM, and the IK11 raised the apoptosis from 4.41% to 26.34% and the necrosis from 3.00 % to 21.44 % at a concentration of 10 μM 24 hours after the IK11 stimulus. These lattest values did not increase more at a concentration of 25μM. We also noticed that the mitochondrial ROS production reached the maximum level 24 hours after the IK11 (10 μM) treatment, while the mitochondrial membrane was depolarized 30 minutes after the IK11 stimulus. Therefore, the IK11 definetely induces the mPTP opening at a concentration of 10 μM and thus triggers the apoptosis and necrosis. This is also confirmed by the literary results which state that the mPTP can cause apoptosis if the mitochondrial permeability transition pore is opened for a shorter time, while if the mPTP is opened for longer it can cause necrosis. Later our results showed that the NAC, which is an excellent antioxidant, completely diminished the reactive oxygen species but had no effect on the variability of HepG2 cells at the concentration of 2mM after the IK11 (10 μM) treatment. At the same time, the trans-resveratrol, which also has an antioxidant property, recovered the cell viability after the IK11 (10 μM) stimulus.The trans-rezveratrol, according to the literature, can inhibit the mPTP opening by inducing the phosphorylation of GSK-3β which

phosphorylates the CypD and thus inhibits the mPTP opening. In the light of our research and the literary results we can conclude that the IK11 induced such strong signal transduction changes that the oxidative stress had only a slight effect on it. At the same time, the resveratrol could decrease the mPTP opening through the signal transduction.

Later we examined whether the IK11 (10 μM) induced the activation of PARP1 and PARP2. PARP1 and PARP2, according to the literature, can trigger the cell death through the inhibition of hexokinase, ATP synthesis and they can also provoke the mPTP opening. These proteins also trigger the cell death by exhausting the ATP and NAD^+ stores while they help to repair the single-strand DNA breaks which can appear due to the oxidative stress. During our experiments we used PJ34 and siRNA PARP inhibitors. According to the literature the PJ34 inhibits the PARP1 and PARP2 enzymes but the siRNA inhibits only the PARP1 protein. Our results showed that the inhibition of PARP1 and/or PARP2 activation almost completely decreased the whole cell ROS production, which indicated the opening of mPTP, and restored the HepG2 cell viability 24 hours after the IK11 (10 μM) treatment.The inhibition of PARP1 and PARP2 with PJ34 significantly decreased the IK11 induced activation of JNK2 and further lowered the IK11 induced decrease of the Akt1 activation after the IK11 stimulus. In the case when we decreased the Akt1 (Ser473) activation with LY294002 (PI3K inhibitor) and Akt-inhibitor IV (Akt inhibitor) inhibitors we saw that the viability of the HepG2 cell just slightly increased after the IK11 treatment. Thus, the Akt1 just slightly contributes to the cell death after the IK11 treatment in HepG2 cells. We can find in the literature that the Akt1 is indispensable for the HepG2 cell division in physiological conditions. Since the PJ34 inhibitor reduced the Akt1 activation in control HepG2 cells we investigated whether the PJ34 decreases the division of HepG2 cells in itself, too. In our studies we found that the PJ34 prevents the cell from entering from the S phase into G2 phase and then into mitosis.Thus the IK11 and PJ34 PARP inhibitor prevented the cell division by inhibiting the activation of Akt1. Later, when we inhibited the JNK2 activation with the SP600125 JNK inhibitor, we found that the viability of the HepG2 cell significantly increased. These results proved that the PJ34 PARP inhibitor increased the cell viability by the inhibition of the activation of JNK2.

Conclusion

In the peritoneal macrophage cells the lack of CypD decreased the mitochondrial ROS production by inhibiting the mPTP opening and prevented the appearance of the extramitochondrial ROS production after the LPS treatment. Thus, the lack of CypD prevented the NF-κB activation, and also the expression of NF-κB induced genes (iNOS, TNF-α). It increased the inactivation the FoxO-1, FoxO3-a, too. These factors are the target proteins of Akt1 kinase. FoxO-1, FoxO3-a reduce the oxidative damage or indicate the apoptosis of the cells if the oxidative damage is severe. The effect of CypD deficiency on the activation of Akt1 was different from the effects of NAC and ferulaldehyde. This is so, because NAC and ferulaldehyde decreased the activation of Akt1 after the LPS treatment in the macrophage. However, the effect of the CypD abligation has the same effect of the PJ34 PARP1 inhibitor and trans-resveratrol, because these substances increased the phosphorylation of Akt1while they decreased the activation of NF-κB activation after the LPS treatment in the macrophages. In the CypD-/- cells the signal transduction changing cannot be explained only by the oxidative signalling. This is due to the activation of Ak1, which increased not only in the LPS-treated cells but also in the control cells compared to the wild-type cells, while the ROS production in these cells did not change. Thus the CypD modified the signal transduction not only through the decreasing of ROS production. It is not yet known what is in the background, but we can read in the literature that the F ATP synthase, which opens inner pores, can modify the signal transduction, too. Besides, the lack of CypD increased the Akt1 activation after the LPS treatment and it evoked the appearance of the M2 phenotype. Thus, the lack of CypD ensured that the pro-inflammatory cytokine secretion and classical activation of the macrophages would not become excessive, and this way it decreased the Th1-tpye cellular adaptive immune response in the early phase of inflammation. Here it should be mentioned that the pro-inflammatory cytokine production also decreased in the lung. Therefore, the lack of CypD provided that the pro-inflammatory cytokine secretion would not become excessive in the hyper-inflammatory phase. Besides, the lack of CypD decreased the immunreaction in the hyper-inflammatory phase, too. This is important because the excessive immune reaction is compensated in the hypo-inflammatory phase with the immunodeficiency, which later causes the death of the patients. Thus, 75% of CypD knockout mice could survive the 40mg/kg LPS treatment, which would be lethal in normal cases. It is also proved by the totally decreased ALI (acute lung injury) histological signs.

During our investigations it became clear that the ferulaldehyde slightly inhibited the mitochondrial depolarization, which indicated the mPTP opening, while it completely decreased the oxidative stress in the (100 ng/ml) LPS-treated RAW264.7 cells.Thus, the antioxidant effect of ferulaldehyde was predominated, and not its ability to inhibit the pores opening to eliminate the all reactive oxygen species 24 hours after the LPS-treatment. The feruladehid inhibited the activation of NF-κB and Akt1 by reducing the oxidative stress. According to the literature its effect was completely consistent with the effect of the antioxidant NAC (10 mM). NAC perfectly eliminated the reactive oxygen species in the (100 ng/ml) LPS-treated RAW264.7 cells and consequently inhibited the activation of NF- NF-κB and Akt1. At the same time the ferulaldehyde modified the Akt1 activation not only through the oxidative signalling, but also by increasing the activation of Akt1 in the control cells, too. According to our examinations it became clear that the ferulaldehyde decreased the activation of MAP kinase not only by decreasing of the oxidative signal transduction but also by increasing the level of MKP1 protein. In the literature we can read that the activated Akt2 and GSK-3β can phosphorylate the CypD and this way it inhibits the mPTP opening. However, the ferulaldehyde inhibited the activation of Akt1, while completely decreased the Akt1 target protein, the GSK-3β kinase phosphorylation 30 minutes after the LPS-treatment. Thus, the phosphorylated GSK-3β could not inhibit the inner pores opening by the phosphorylation of CypD. Over and above probably not only the Akt2 could be responsible for the phosphorylation of GSK-3β, but also the Akt1. In summary, the ferulaldehyde prevented the activation of Akt1 which is necessary for the cells to abolish the classical activation of macrophages and which triggers M2 phenotype switching. This result could partly explain our earlier result that the ferulaldehyde decreased the LPS-induced inflammation processes in mice only in the early stage, and did not have any effects on their survival.

During the experiments it became clear that mPTP opening is absolutely required for the apoptotic and necrotic cell, and PARP1 and PARP2 regulate the mPTP opening in the IK11-treated HepG2 cells. Besides, the results showed that the inhibition of PARP(1,2) further decreased the activation of Akt1 after the IK11 treatment, which is responsible for the cell division. At the same time this inhibitors decreased the IK11 induced JNK2 activation, which is responsible for the cell viability. Because the activation of Akt1 was under the control level in the HepG2 cells, the PJ34 PARP inhibitor could not decrease the inner pores opening by the phosphorylation of CypD through the PI3K/Akt1/GSK-3β pathway. At the same time JNK2

could provoke the opening of outer pores after the IK11 treatment. This assumption is based on the articles, which are about the fact that JNK1 can induce the outer pores opening and thus we think that probably JNK2 has the same effect. According to our results we can conclude that PARP inhibitors prevented the exhaustion of the ATP stores, which is important for the increased cell division and the metabolism of cancer cells. This happened due to the prevention of the glycolysis, the inhibition of the mPTP opening, and the maintainence of the efficient work of the electron transport chain and NAD+, ATP stores. Our result can be definetely explained with the effect of PARP1 on the gene expression and this way on the signalling process that trigger the mPTP opening. PARP1 shows its effect by being able to poly-ADP-ribosylate the histone proteins, and thus it induces the decondensation of chromosomes and the expression of genes.

In our earlier experiments we found that the ferulaldehyde decreased inflammation only in the early phase, and in the long run it didn't ensure the survival of the mice. In contrast to this the abligation of PARP1 decreased the death of mice to 90%. We learned about the PARP1 activation that it is indispansible for the opening of MPTP in IK11-treated HepG2 cells. However, the inhibition of PARP1 was effective only in the mice model.

Thus, the development of an inhibitor for the isomerase function of CypD could be a good solution for sepsis. There is an increasing need for this inhibitor since such multiresistant microorganisms appear more and more frequently which could trigger sepsis. Moreover, it could be a solution for every disease, which is characterized by inflammation, excessive oxidative stress and mitochondrial regulated cell death, for example Alzheimer, muscular distrophy, autoimmune diseases, ischemia-reperfusion induced myocytes death, etc.

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