Cytoprotective Role of PARP inhibition, Akt Activation and Mitochondrial Protection in Oxidative Stress

Ph.D. thesis

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Introduction:

Under several pathological conditions, reactive oxygen species-induced damages play important roles in pathogenesis. High levels of reactive oxygen species are generated from a variety of sources such as the xanthine oxidase system, the leakage of electrons from the mitochondrial respiratory chain, the cyclooxygenase pathway of arachidonic acid metabolism, and the respiratory burst of phagocyte cells, and they can cause DNA damage-generating singlestranded DNA breaks. Poly(ADP-ribose)polymerase (PARP-1,2 EC 2.4.2.30) is a multifunctional nuclear enzyme that is activated by DNA strand breaks and catalyzes the covalent coupling of branched chains of ADP-ribose units to various nuclear proteins such as histone proteins and PARP-1 itself. PARP-1 is involved in chromatin remodelling, DNA repair, replication, transcription, and the maintenance of genomic stability by, in part, poly(ADP-ribosyl)ation. With moderate amounts of DNA damage, PARP-1 is thought to participate in the DNArepair process. However, oxidative stress, which induces a large amount of DNA damage, can cause excessive activation of PARP-1, leading to depletion of its substrate NAD^+; and in an effort to resynthesize NAD^+, ATP is also depleted, resulting in cell death as a consequence of energy loss. PARP inhibitors show pronounced protection against myocardial ischemia, neuronal ischemia, acute lung inflammation, acute septic shock, zymogen-induced multiple organ failure, and diabetic pancreatic damage, providing evidence for the role of excessive PARP-1 activation in cell death. It is believed that by preventing excessive NAD^+ and ATP utilization, PARP inhibitors protect cells against oxidative damage, but some recent data suggest a more complex mechanism for the cytoprotection.

Amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride) is a class III antiarrhythmic agent used in the clinical practice for the treatment of various arrhythmias. We found that it presented a protective effect on the postischemic heart by enhancing the recovery of high-energy phosphate metabolites and inhibiting mPT at low concentrations. However, when administrated at higher concentrations, it induced mitochondrial swelling, the collapse of the mitochondrial membrane potential ($\Delta\psi$) and apoptosis.

Desethylamiodarone, the major metabolite of amiodarone, also has antiarrhythmic activity, significantly increasing the action potential duration (class III antiarrhythmic effect) and decreasing the maximum rate of depolarization (class I antiarrhythmic effect) at clinically relevant concentrations. Desethylamiodarone rapidly accumulates in the lung after amiodarone treatment, sometimes in higher concentrations than amiodarone itself raising the possibility that this metabolite contributed to the effects of amiodarone. However, we found it to be more toxic than amiodarone in pulmonary cell types, suggesting that desethylamiodarone may play an important role in the development of the amiodarone treatment-induced pulmonary fibrosis.

The cardioprotective effect of amiodarone is due, at least in part, to the inhibition of mitochondrial permeability transition (mPT) at lower concentrations. However, when administrated in higher concentrations, it induced mitochondrial swelling as well as the collapse of the mitochondrial membrane potential ($\Delta\psi$). Desethylamiodarone, the major metabolite of amiodarone —reported by some authors to be the major cause of the amiodarone administration induced toxicity— does not inhibit mPT at any concentration, and induce swelling and the collapse of the membrane potential at higher concentrations. The difference between the effect of amiodarone and desethylamiodarone is due to the absence of an ethyl side chain from the amino group of desethylamiodarone. This led us to the conclusion that the structural modification of amiodarone can improve its inhibitory effect on mPT as well as its beneficial effect in ischemia- reperfusion injuries. Considering its advantages, a lot of effort has been made in the past decade to improve pharmacokinetic properties of amiodarone, mainly by chemical alterations of the original molecule such as synthesis of monoiodo derivatives, introduction of carboxymethoxy side chain instead of tertiary amine or substitute the original n-butyl group for an isobutyl ester.

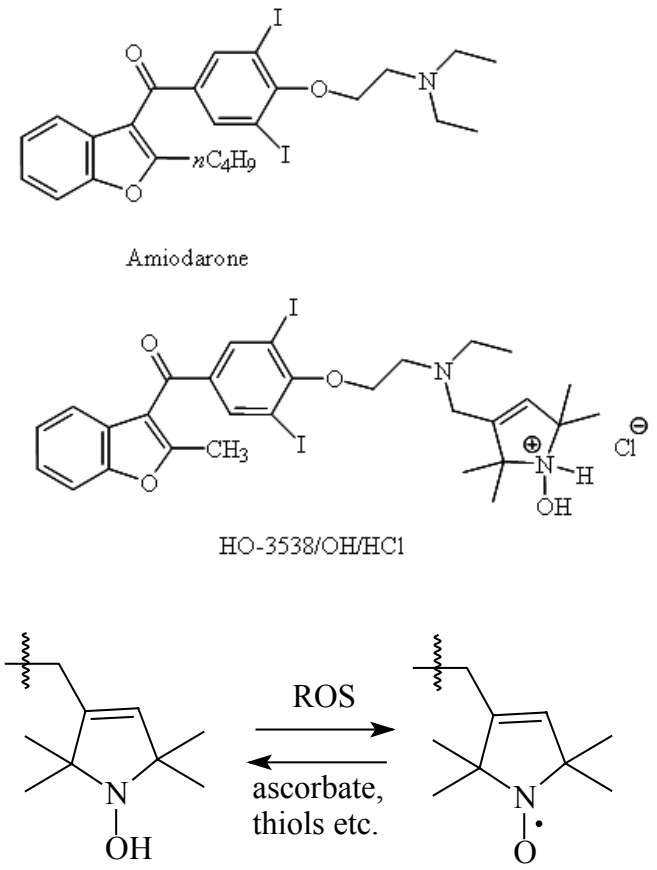

Fig. 1. The chemical structures of amidarone HO-3538 and SCAV

The chemical structures of amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride), HO-3538 (2-Methyl-3-(3,5-diiodo-4-{2-[N-ethyl,N-(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl) ethyl]}oxybenzoyl)benzofurane· 2HCl salt). The 1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl component (SCAV) of HO-3538 possesses ROS scavenging activity. The oxidized form can be reduced by thiols, ascorbate, GSSH, etc.

Prof. Hideg and his co-workers provided numerous paramagnetic and diamagnetic amiodarone derivates synthesized by them for screening their effect on the mitochondrial permeability transition. In this study, we analyzed the effect of HO-3538 (Fig.1), the most effective novel amiodarone analogue, in which an ethyl side chain is substituted with 1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl (SCAV) that has been described to possess free-radical scavenging activity on the permeability transition *in vitro* and in cultured cells as well as on ischemia-reperfusion in Landendorff-perfused rat hearts. This amiodarone analogue can form the basis of developing novel amiodarone

analogues that have the same or enhanced efficiency as amiodarone but with fewer side effects.

Objectives.

1. Several study have shown that the different PARP inhibitors improve the survival of cells in oxidative stress. Is this cytoprotection achieved via direct attenuation of PARP-1 activation or decrease of the activity of other ADP-ribosylating enzymes?
2. It is widely accepted fact that the cytoprotective effects of PARP inhibition in oxidative stress are based on the prevention of NAD^+ and ATP depletion. Is this protection mediated only through the preservation of the energetics of cells alone or there might be present other mechanisms as well?
3. Our previous works demonstrated that amiodarone has a biphasic effect on mPT; it protected the mitochondria from mPT at low concentration although it induces a CsA independent mitochondrial swelling at higher concentration. Can appropriate substitution of amiodarone suppress the mPT inducing effects of the drug while maintaining its protective effects on mitochondria?
4. After screening of many amiodarone derivates we selected a novel amiodarone analogue (HO-3538), in which an ethyl side chain was substituated with a SOD-mimetic one. What are the beneficial effects of the combination of mPT inhibition and free-radical scavenging in ischemia-reperfusion and oxidative stress?

Materials and Methods.

Materials

PI3-kinase inhibitors LY 294002 and wortmannin, PARP-1 inhibitor PJ-34, protease inhibitor mixture, and all of the chemicals for cell cultures were purchased from Sigma. Fluorescent dyes JC-1, fluorescein-conjugated annexin V, and propidium iodide were from Molecular Probes. The following antibodies were used: anti-phospho- Akt (Ser473) and anti-phospho-GSK3_(Cell Signaling Technology, Beverly, MA); anti-PAR and anti-PARP (Alexis Biotechnology, London, U.K.); anti-actin, anti-mouse IgG, and anti-rabbit IgG (Sigma). Cyclosporin Awas from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA); rhodamine 123 (Rh123) and carboxy-H2DCFDAwere from Molecular Probes (Eugene, OR, USA), anti-cytochrome c monoclonal antibody was from Pharmingen (San Diego, CA, USA), anti-apoptosis-inducing factor (AIF) polyclonal antibody was from Oncogene (San Diego, CA, USA), HO-3538 (2-methyl-3-(3,5-diiodo-4-{2-[Nethyl, N-(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol- 3-ylmethyl) ethyl]}oxybenzoyl)benzofurane · 2HCl salt) was produced as described previously [27] all other compounds were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Cell Culture

WRL-68 human liver cells, H9C2 mouse cardiomyoblast, Jurkat cells were from the American Type Culture Collection (Wesel, Germany). The cells were maintained as monolayer adherent culture in minimum Eagle's medium, Dulbecco's modified Eagle's medium containing 1% antibiotic-antimycotic solution and 10% fetal calf serum (MEM/ FCS) in a humid 5% CO2 atmosphere at 37 °C. Jurkat cells were maitained in RPMI.

Transdominant Expression of the DNA-binding Domain of PARP

The coding region of the N-terminal DNA-binding domain of PARP (PARP-N214, amino acid residues 1–214 (34)) was amplified by PCR and cloned in-frame into pEGFP-C1/N3 vectors (Clontech) after cutting with HindIII and EcoRI restriction enzymes (Fermentase, Vilnius, Lithuania). For enabling active nuclear transport of the green fluorescent protein (GFP)-tagged PARP-N214, the nuclear localization signal was added

to the N terminus of the PARP-N214 sequence using PCR primers coding for the nuclear localization signal sequence. The recombinant pPARPGFP-C1/N3 vectors were purified by a plasmid purification kit (Qiagen, Valencia, CA) and utilized for transient transfection of WRL-68 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For an effective transdominant expression of PARP-DBD, the transfection step was repeated 48 h after the first transfection, and the experiments on the cells were performed 40 h after the second transfection. *Suppression of PARP-1 Expression by siRNA Technique*—WRL-68 cells were transiently transfected with siRNA designed for PARP 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.

Cell Viability Assay

The cells were seeded into 96-well plates at a starting density of 104 cell/well and cultured overnight beforeH2O2 and different inhibitors modulating the effect of theH2O2 were added to the medium at a concentration and composition indicated in the figure legends. After 3 h of treatment, the medium was removed, and fresh MEM/FCS containing 0.5% of the water-soluble yellow mitochondrial dye MTT was added. Incubation was continued for an additional 3 h, and the MTT reaction was terminated by adding HCl to the medium at a final concentration of 10 mM. The amount of water-insoluble blue formasan dye formed from MTT was proportional to the number of live cells and was determined with an Anthos Labtech 2010 enzyme-linked immunosorbent assay reader at 550 nm wavelength after dissolving the blue formasan precipitate in 10% SDS. All experiments were run in at least four parallels and repeated three times.

Western Blot Analysis

The cells were seeded and treated as for the cell viability assay. After 1 h of treatment, the cells were harvested in a chilled lysis buffer of 0,5 mM sodium metavanadate, 1 mM EDTA, and protease inhibitor mixture in phosphate-buffered saline. The proteins were

precipitated by trichloroacetic acid, washed three times with _20 °C acetone, and subjected to SDS-PAGE. Proteins (30 _g/lane) were separated on 12% gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% low fat milk for 1 h at room temperature, then exposed to the primary antibodies at 4 °C overnight at a dilution of 1:1,000 in blocking solution. Appropriate horseradish peroxidase-conjugated secondary antibodies were used for 2 h at room temperature and a 1:5,000 dilution. Peroxidase labeling was visualized with enhanced chemiluminescence (ECL) using an ECL Western blotting detection system (Amersham Biosciences). The developed films were scanned, and the pixel volumes of the bands were determined using NIH Image J software. All experiments were repeated four times.

Fluorescent Microscopy

Wild type or transfected WRL-68 cells were seeded to poly-L-lysine-coated (2.5–5 μg/cm2) glass coverslips and cultured at least overnight before the experiment. After subjecting the cells to the appropriate treatment (indicated in the figure legends), the coverslips were rinsed twice in phosphate-buffered saline then placed upside down on the top of a small chamber formed by a microscope slide and a press-to-seal silicone isolator filled with phosphate-buffered saline containing 4.5 g/liter glucose and 20 mM HEPES pH 7.4. Cells were imaged with an Olympus BX61 fluorescent microscope equipped with a ColorView CCD camera and analySISR software using a 60 _ objective and epifluorescent illumination. For GFP fluorescence, 450– 490 nm excitation and _520 nm emission (green) filters were used. For JC-1 fluorescence, the cells were loaded with the dye for 10 min, then the same microscopic field was imaged first with 546 nm bandpass excitation and _590 nm emission (red), then with green filters. Under these conditions we did not observe considerable bleed-through between the red and green images.

Animals

Wistar rats were purchased from Charles River Hungary Breeding Ltd. (Budapest, Hungary). The animals were kept under standardized conditions; tap water and rat chow were provided ad libitum. Animals were treated in compliance with approved institutional animal care guidelines.

Mitochondrial permeability transition

The mPT was monitored by following the accompanying large amplitude swelling via the decrease in absorbance at 540 nm [31] measured at room temperature by a Perkin–Elmer fluorimeter (London, UK) in reflectance mode. Briefly, mitochondria at the concentration of 1 mg protein/ml were preincubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, 0.5 mM phosphate) containing the studied substances for 60 s. Mitochondrial permeability transition was induced by the addition of 60 μM Ca2+ or amiodarone, or HO-3538 at the indicated concentration. Decrease in E540 was detected for 20 min. The results are illustrated by representative original registration curves from at least five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts.

Mitochondrial membrane potential

The membrane potential was monitored by fluorescence of Rh123, released from the mitochondria after the induction of permeability transition at room temperature by using a Perkin–Elmer fluorimeter at an excitation wavelength of 495 and an emission wavelength of 535 nm. Briefly, mitochondria at the concentration of 1 mg protein/ml were preincubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, 0.5 mM phosphate) containing 1 μM Rh123 and the studied substances for 60 s. Alteration of the mitochondrial membrane potential was induced by the addition of HO-3538 at the indicated concentration. Changes in fluorescence intensity were detected for 4 min. The results are illustrated by representative original registration curves from five 837 Z. Bognar et al. / Free Radical Biology & Medicine 41 (2006) 835–848 independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts.

Determination of cytochrome c level by high-pressure liquid chromatography (HPLC)

The analysis of cyt-c was performed on a nonporous 33 × 4.6-mm KOVASIL-MS C18 column (Zeochem AG, Uetikon, Switzerland). Separations were carried out on a Dionex

HPLC system consisting of a Dionex P 580 lowpressure gradient pump and a Dionex UVD 340S diode array detector (chromatograms were detected at 393 nm). The samples were injected by a Rheodyne 8125 injector equipped with a 20-µl loop. Instrument control and data acquisition were carried out using Chromeleon data management software. The measurements were accomplished using gradient elution. Eluent A consisted of 10:90 acetonitrile:water + 0.1% (v/v) trifluoroacetic acid and eluent B consisted of 90:10 acetonitrile: water + 0.1% (v/v) trifluoroacetic acid. The applied gradient program was the following: 0 to 7 min, from 0% B to 70% B; 7 to 12 min, from 70% B to 100% B; 12 to 12.5 min, from 100% B to 0% B; 12.5 to 14.5 min, 0% B. The flow rate was 1 cm3 · min−1. The column reequilibration was involved in the gradient program. Data acquisition was performed from at least three independent experiments.

Detection of antioxidant effect in cells

H9C2 cells were seeded into 96-well plates at a starting density of 2 × 104 cells/well and cultured overnight. The next day, the cells were exposed for 90 min to 1 mM H2O2 followed by two washings with phosphate-buffered physiological saline solution. The cells were then preincubated for 30 min in fresh medium containing HO-3538 or different antioxidants at the concentration of 10 µM, and 1 µM carboxy-H2DCFDA was added to the medium for a further 1 h incubation. Fluorescence of carboxy-DCFDA oxidized stoichiometrically by the ROS was measured by using a fluorescence ELISA reader (BMG Laboratories, Offenbach, Germany) at excitation and emission wavelengths of 485 and 555 nm, respectively. All experiments were run at least four in parallel and repeated three times.

Heart perfusion

Hearts were perfused via the aorta as described before [12] in the absence or presence of different concentrations of HO-3538. After being washed (nonrecirculating period of 15 min), hearts were perfused under normoxic conditions for 10 min; the flow was subsequently discontinued for 30 min by inflating a balloon (ischemia), which was followed by 15 min of reperfusion. HO-3538 at the indicated concentrations was added at the beginning of the normoxic perfusion phase. Levels of high-energy phosphate

intermediates were monitored in the magnet of a 31P NMR spectroscope during the entire perfusion.

NMR spectroscopy

NMR spectra were recorded with a Varian UNITYINOVA 400 WB instrument. 31P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z d SPEC 20-mm broadband probe (Nalorac Co., Martinez, CA, USA), applying WALTZ proton decoupling ($\gamma B2$ = 1.6 kHz) during the acquisition only. Field homogeneity was adjusted by following the 1H signal (w1/2 = 10–15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free induction decay. Flip-angle pulses of 45° were used after a 1.25-s recycle delay, and transients were acquired over a 10-kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16,384. Under the above conditions, relative concentrations of the species are proportional to the corresponding peak areas, because interpulse delays exceeded four to five times the T1 values of the metabolites that were analyzed in the 31P experiments. Data were acquired from five independent experiments for each concentration of HO-3538.

Statistical analysis

Data are presented as means ± SEM. For multiple comparisons of groups ANOVAwas used. Statistical difference between groups was established by paired or unpaired Student's t test, with Bonferroni correction.

Conclusions

1. Several studies demonstrated that the different pharmacological PARP inhibitors have got beficial effects in oxidative stress. We provided evidence that suppression of PARP-1 activation by small molecular weight inhibitor, by siRNA method, or by the transdominant expression of PARP-DBD protected cells from oxidative stress. Since the sequence of the siRNA and the PARP-DBD were based on the sequence of the nuclear PARP-1, the same cytoprotection by the pharmacological and non-pharmacological inhibition has proven that inhibition of the single-stranded DNA break-induced PARP-1 activation was responsible for the protective effect in oxidative stress rather than some side effect of the pharmacological inhibitor.
2. We provided evidence for undermining the classical view that cytoprotection by PARP inhibitors relies exclusively on the preservation of NAD^+ and consequently the ATP stores in oxidative stress. Inhibition of Akt activation by specific inhibitors in a significant extent counteracted the cytoprotective effect of PARP inhibitor, indicating that the PARP inhibition-induced Akt activation was very significantly responsible for the cytoprotective property of PARP inhibitors. We established that the benefit of PARP inhibition is mediated through two different processes in oxidative stress: the preservation of energetics of cells and activation of PI3K/Akt as a well-known survival signaltrasduction pathway.
3. We synthesized numerous paramagnetic and diamagnetic amiodarone derivates and screened their effects on the mitochondrial permeability transition. We found an amiodarone analgue HO-3538 in which an ethyl side chain is substituted with 1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl, which has been described to possess SOD-mimetic activity. HO-3538 completely inhibited the Ca2+-induced swelling at low concentration furthermore did not induce mPT at higher concentration.
4. HO-3538, although had a less pronounced free-radical scavenging activity than its SOD-mimetic side chain, and some antioxidants, due to its mPT inhibitory activity combined to its SOD-mimetic activity, was much more effective protective effect in ischemia-reperfusion as well as in oxidative stress than any

other compounds tested including amiodarone. This proved that combined mPT inhibitory and free-radical properties whithin the same molecule might represent a useful approach in the therapy of oxidative damage related diseases.

List of Publications

Publications supporting the dissertation:

Antal Tapodi, Balazs Debreceni, Katalin Hanto, Zita Bognar, Istvan Wittmann, Ferenc Gallyas, Jr.,Gabor Varbiro, and Balazs Sumegi: Pivotal role of Akt activation in mitochondrial protection and cell survival by poly(ADP-ribose)polymerase-1 inhibition in oxidative stress***.*** ***J Biol Chem.*** 2005 Oct 21;280(42):35767-75.

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