AGENTS AFFECTING THE APOPTOTIC PROCESS BY INFLUENCING THE MITOCHONDRIAL PERMEABILITY TRANSITION.

Ph.D. Theses

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Introduction.

General features of programmed cell death.

Apoptosis, or programmed cell death is recognized as a critical element in the removal of cells following exposure to toxic compounds as well as during development and in degenerative disorders. In general programmed cell death represents a continuum of cell death ranging from classical apoptosis to necrosis at its two poles. Apoptosis is a common and evolutionary conserved mechanism present in most living organism. Tissue homeostasis relies on the tightly controlled removal of superfluous, damaged and ectopic cells through apoptosis. Whereas an aberrant resistance to apoptosis participates in the development of neoplasia, excessive cell death, through apoptosis contributes to acute organ failure as well as to chronic diseases involving the loss of post-mitotic cells.

Apoptosis has two distinct pathways. The *intrinsic cell death pathway* involves the initiation of apoptosis as a result of a disturbance of intracellular homeostasis. In this pathway, mitochondria are critical for the execution of cell death, and so this pathway has been referred to as the mitochondrial cell death pathway. The *extrinsic pathway* involves the initiation of apoptosis through ligation of plasma membrane DRs (deaty receptors), and so this pathway is also referred to as the DR pathway. While the initiation mechanism of these pathways are different, both converge to result ultimately in cellular morphological and biochemical alterations characteristic of apoptosis. It is also apparent that a considerable interaction between the pathways occur upstream of the convergence point, and that individual cells possess a considerable degree of redundancy in their apoptotic pathways.

The role of mitochondria in apoptosis.

The *intrinsic cell death pathways* follow from proapoptotic signals resulting in a disruption of intracellular homeostasis, that is the signals for cell suicide originate within the cell (Green

and Reed, 1998; Wang, 2001). The mitochondria are the primary intracellular initiation sites although the endoplasmatic reticulum has also been implicated. All cells harbor the latent ability to undergo programmed cell death. Under normal circumstances, apoptosis is suppressed, as a result of the rigorous compartmentalization of catabolic enzymes and their activators. Mitochondria play a major role in this subcellular partitioning of death-regulating biochemical signals (Penninger and Kroemer, 2003). For example, cytochrome *c* is confined to the mitochondrial intermembrane space preventing it from interacting with apoptotic-protease-activating factor 1 (Apaf-1), a cytosolic protein. Upon permeabilization or rupture of the outer mitochondrial membrane, cytochrome *c* binds to Apaf-1, leading to allosteric activation of procaspase-9. Caspase-9 then proteolytically activates caspase-3, one of the principal proteases that participates in the execution of cell death. Similarly, Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein of low isoelectric point [pI]) and Omi/HtrA2, two intermembrane proteins, are normally physically separated from cytosolic inhibitors of apoptosis proteins (IAPs). Upon mitochondrial permeability transition (MPT), Smac/DIABLO and Omi/HtrA2 neutralize IAPs, and thus relive the IAP-mediated inhibition caspase-3 and –9. Two DNA-destroying enzymes, apoptosis inducing factor (AIF) and endonuclease G, are also normally confined to the mitochondrial intermembrane space, but following MPT they can move to the nucleus where they mediate chromatinolysis.

Mitochondrial Permeability Transition (MPT).

MPT is a major event in physiological as well as pathological cell death, and not surprisingly, it is regulated at multiple levels (Zamzami et al., 1996). MPT can be induced by multiple pro-apoptotic second messengers (Fig. 1.), including Ca^{2+}, reactive oxygen species (ROS), lipid messengers (e.g. ceramide and ganglioside GD3) and stress kinases. In addition it is facilitated by pro-apoptotic proteins from the Bcl-2 family, and is inhibited by anti-apoptotic

Bcl-2-like proteins. MPT might involve the formation of protein-permeable pores by oligomers of Bax and Bak (two pro-apoptotic proteins of the Bcl-2 family), as well as the transient or permanent opening of a variety of different channels, including those contained in the permeability transition pore complex (PTPC), such as the voltage-dependent anion channels (VDAC) in the outer membrane and the adenine-nucleotide translocase (ANT) in the inner membrane. In addition to PTPC-dependent mechanisms, recent findings suggest that apoptotic proteins such as cytochrome *c* can be released from the mitochondria by as yet undetermined mechanisms that do not involve formation of PTPC. Furthermore, different K^+-selective channels in the inner membrane might control the volume of the mitochondrial matrix and ultimately the intactness of mitochondrial membranes. Moreover, inner-membrane uncoupling proteins (UCPs), which regulate the transmembrane proton gradient and the production of ROS and ATP, are increasingly being recognized for their roles in modulating cell death. Irrespective of the exact mechanism of MPT, it appears that this event can mark the 'point of no return' in the cell death process.

The mechanism of mitochondrial permeability transition (MPT).

Mitochondria are organelles with two well-defined compartments: the matrix, surrounded by the inner membrane (IM), and the intermembrane space, surrounded by the outer membrane (OM). The IM is folded into numerous christae, which greatly increases its surface area. It contains the protein complexes from the electron transport chain, the ATP synthase and the adenine nucleotide translocator (ANT). To function properly, the IM is almost impermeable to the various metabolites in physiological conditions (except for the those that have regulated transport mechanism), thereby allowing the respiratory chain to create an electrochemical gradient ($\Delta\Psi$). The $\Delta\Psi$ results from the respiration-driven, electron-transport-chain-mediated pumping of protons out of the inner membrane and is indispensable for driving the ATP synthase which phosphorylates ADP to ATP. ATP is then exported in exchange for ADP by

the ANT. The OM in normal conditions is permeable to solutes up to about 5,000 Da. Thus, whereas the matrix space contains a highly selected set of small molecules, the intermembrane space is chemically equivalent to the cytosol with respect to low-molecular-weight solutes. The OM permeabilization involves the release of proteins which are normally confined to the intermembrane space, including cytochrome c, Smac/DIABLO, Omi/HtrA2 and AIF. The IM permeabilization may occur in a 'step-wise' manner (Green and Reed 1998; Bernardi 1999), with increasing permeability of solutes up to about 1,500 Da (Fig. 2.), and is manifested as the dissipation of the proton gradient responsible for the trans-membrane potential ($\Delta\Psi$), an extrusion of small solutes (such as calcium or glutation), or an influx of water and sucrose (which, in sucrose-containing media, leads to large-amplitude swelling of the matrix).

Objectives

Numerous drug used in human therapy possesses toxic effect on tissues and cells. Since the mitochondria is involved in both the apoptotic and the necrotic cell death process, we analyzed effect of well known toxic drugs on the mitochondrial functions. Taxol is a widely used anti-tumor agent, that was described to induce apoptosis, therefore we examined the direct mitochondrial effect of Taxol to elucidate the role mitochondria plays in the apoptosis induced by this drug. Amiodarone is the antiarrhythmic drug of choice in case of the most severe arrhythmias, with excellent antiarrhythmic properties. However it has serious side effects limiting its clinical use. We analyzed the role mitochondria plays in the beneficial cardiac and toxic extracardiac effect of amiodarone and its major metabolite, N-desethylamiodarone.

Materials and Methods.

Materials. Paclitaxel was from ICN Biomedicals Inc.; Taxol was from Bristol Myers Squibb; CsA was from Biomol Research Labs. Inc. ; rhodamine123 (Rh123), dihydrorhodamine123 (DRh123) and N-acetyl-8-dodecyl-3,7-dihydroxyphenoxazine (resorufin) were from Molecular Probes; amiodarone was from Chinoin Sanofi; all other compounds were from Sigma Chemical Co.

Animals. Wistar rats kept under standardized conditions; tap water and rat chow were provided ad libitum. Animals were treated in compliance with approved institutional animal care guidelines.

Cell culture. PANC-1 human pancreatic epithelioid carcinoma cells, BRL 3A rat liver cells, HepG-2 human hepatocellular carcinoma, WRL 68 human liver cells, H9C2 mouse cardiomyocytes and Sp-2 mouse myeloma cells were from American Type Culture Collection.

Isolation of mitochondria. Rat liver and heart mitochondria were prepared according to standard protocol.

Mitochondrial oxygen consumption was assessed by a Clark electrode. The mitochondrial respiration was measured in the presence of different concentrations of amiodarone or desethylamiodarone.

Mitochondrial permeability transition was monitored by following the accompanying large amplitude swelling via the decrease in absorbance at 540 nm measured by a Perkin-Elmer fluorimeter in reflectance mode.

Enzymatic permeability transition assay. Measuring of citrate synthase (EC 4.1.3.7) and carnitine acyl transferase (EC 2.3.1.7) activity after the induction of permeability transition was performed as described previously.

Alterations in the mitochondrial membrane potential following the induction of permeability transition was monitored by fluorescence of Rh123, released from the mitochondria and detected by a Perkin-Elmer fluorimeter at an excitation wavelength of 495 and an emission wavelength of 535 nm.

The determination of ROS-formation. ROS formation was detected by the fluorescence of Rh123 formed by ROS-induced oxidation of the non-fluorescent DRh123 in situ at an excitation wavelength of 495 nm and an emission wavelength of 535 nm The ROS-induced oxidation of N-acetyl-8-dodecyl-3,7-dihydroxyphenoxazine forms N-acetyl-8-dodecyl-resorufin (resorufin) which exhibits strong red fluorescence at the excitation wavelength to 578 nm and the emission wavelength to 597 nm. This product is well retained in living cells and organelles by virtue of its lipophilic tail, making it possible to detect ROS production in the lipid phase.

Cell viability assay. PANC-1, BRL-3A, WRL 68 and H9C2 cells were seeded into 96-well plates at a starting density of 2.5 x 10^4 cell/well and cultured overnight. The following day, amiodarone at the indicated concentrations was added to the medium. Forty-eight hours later, 0.5 % of the water soluble dye, MTT^+ was added. Incubation was continued for 3 more hours, the medium was removed, and the water insoluble blue formasan dye formed stochiometrically from MTT^+ was solubilized by acidic isopropanol. Optical densities were determined by an ELISA reader.

Heart Perfusion. Rats weighing 350 g were pretreated with amiodarone or desethylamiodarone exactly as described previously. Hearts were perfused via the aorta. After a washout (non-recirculating period of 15 min), hearts were perfused under normoxic conditions for 10 minutes; the flow was subsequently discontinued for 30 minutes by inflating a balloon (ischemia), which was followed by 15 minutes of reperfusion. Levels of high-

energy phosphate intermediates were monitored in the magnet of a ^{31}P NMR spectroscope during the entire perfusion.

NMR Spectroscopy. NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB instrument as described. Data were acquired from 5 independent experiment for sham-operated and amiodarone-treated groups each.

Statistical analysis. Data were presented as means ± S.E.M. For multiple comparisons of groups ANOVA was used. Statistical difference between groups was established by paired or unpaired Student's *t* test, with Bonferroni correction.

Results.

Paclitaxel induces MPT and dissipation of $\Delta\Psi$ in isolated mitochondria. In a concentration dependent manner, paclitaxel induced swelling in isolated liver mitochondrion, in the presence of low concentration of Ca^{2+} (2.5 μM). Paclitaxel at a concentration of 20 μM induced swelling of mitochondria, and was prevented completely by 2.5 μM CsA. Twenty μM paclitaxel caused the dissipation of $\Delta\psi$, and was also inhibited by 2.5 μM CsA. The opening of the permeability transition pore was also demonstrated by a simple enzyme assay. Citrate synthase and carnitine acyltransferase activity become measurable using externally added acetyl-coenzyme A after the addition of either Ca^{2+} or paclitaxel showing the opening of the permeability pore. This effect was inhibited by 2.5 μM CsA. Release of cytochrome c from the mitochondrial intermembrane space following permeability transition induced by either 150 μM Ca^{2+} or by 20 μM paclitaxel was detected by Western blotting. The degree and slope of the swelling differed greatly among the different tissues, with the highest value for liver, a lower value for kidney and heart, a value close to the detection limit for brain. There

were differences in the degree and slope of the Rh123-release among the different tissues similar as in the case of swelling.

Paclitaxel induces dissipation of $\Delta\Psi$ in cell line. Confocal microscopy was used to monitor the changes in Rh123 and PI fluorescence images of BRL-3A cells. The cationic dye, Rh123 is retained by the mitochondria and shows green fluorescence image of the mitochondria when $\Delta\psi$ is intact, but is released into the cytosol and shows a weak background fluorescence when $\Delta\psi$ is disrupted in consequence to permeability transition. On the other hand, PI is excluded by intact cell membranes, but shows red fluorescent image of the nucleus when cellular energy charge drops to zero or the integrity of the cell membrane is damaged. Representative fluorescent images taken 45 minutes after the addition of paclitaxel and CsA demonstrate the effect of these drugs on the cells.

Effect of amiodarone and desethylamiodarone on Ca^{2+}-induced MPT in isolated rat liver mitochondria. In isolated liver mitochondria, the swelling induced by Ca^{2+} was completely inhibited by 2.5 μM of CsA. Depending on its concentration, amiodarone had a *biphasic* effect on mitochondrial swelling. Up to the concentration of 10 μM, amiodarone inhibited the rapid swelling induced by Ca^{2+} in a concentration dependent manner. At higher concentrations, amiodarone proved to be less effective in delaying the Ca^{2+}-induced swelling. In contrast to amiodarone, desethylamiodarone did not show any inhibitory effect on the mitochondrial permeability transition induced by Ca^{2+} up to the concentration of 10 μM.

At concentrations above 10 μM, amiodarone induced mitochondrial swelling by its own, that was not inhibited by 2.5 μM of CsA. At the concentration of 30 μM desethylamiodarone, induced swelling with a rate, more pronounced than equimolar concentration of amiodarone, and was not inhibited by 2.5 μM of CsA either. The effect of amiodarone or desethylamiodarone on isolated rat heart mitochondria was basically the same.

The opening of the permeability transition pore was also detected by an enzyme assay decribed previously

Effect of amiodarone and desethylamiodarone on dissipation of $\Delta\Psi$ in isolated liver mitochondria. Sixty μM Ca^{2+} caused the dissipation of $\Delta\Psi$. When the mitochondrial membrane was depolarized by Ca^{2+} in the presence of 2.5 μM CsA, after a transient depolarization lasting for about a minute, $\Delta\Psi$ returned to the value identical to the one before the addition of Ca^{2+}. Ten μM of amiodarone depolarized the mitochondrial membrane in a similar extent as did the 60 μM of Ca^{2+}, and, its depolarizing effect was not influenced at all by 2.5 μM of CsA. Amiodarone caused a concentration dependent release of Rh123 from liver mitochondria. In contrast to this, desethylamiodarone, up to the concentration of 10 μM did not induce the dissipation of $\Delta\Psi$, However, at the concentration of 20 μM it caused Rh123 release similar as did 20 μM of amiodarone, 60 μM of Ca^{2+}, or as did 1 μM of FCCP. The depolarizing effect of 20 μM of desethylamiodarone was not influenced at all by 2.5 μM of CsA.

Paclitaxel induces ROS formation in isolated rat liver mitochondria. Since ROS formation can induce the mitochondrial permeability transition, we studied the effect of paclitaxel on ROS production by monitoring the fluorescence of Rh123 or resorufin oxidized by the ROS. By virtue of its dodecyl group, resorufin was localized in membranous regions and detected ROS formation in lipid phase while Rh123 reflected to ROS levels in aqueous phase. Paclitaxel induced ROS-formation in isolated liver mitochondria in a concentration dependent manner. A much more intense ROS-formation was detected by resorufin than by Rh123. Selective inhibitors of the respiratory complexes caused a transient fast increase of the mitochondrial ROS formation followed by a plateau where the rate of ROS formation was the same as before the addition of the given substance. When added on this plateau, paclitaxel induced ROS formation with a time-course similar to that observed in the absence of the

inhibitors of the respiratory chain. However, KCN and other inhibitors of cytochrome oxidase, azide and NO, inhibited the paclitaxel-induced ROS production.

The effect of amiodarone and desethylamiodarone on ROS formation in isolated rat mitochondria. Up to the concentration of 100 μM, amiodarone and desethylamiodarone did not induce ROS formation in mitochondria isolated from either liver or heart as detected by Rh123 or resorufin.

Effect of paclitaxel on mitochondrial oxygen consumption. Since inhibitors of the respiratory chain caused a transient ROS formation, we checked whether paclitaxel had inhibitory effect on mitochondrial respiration. Oxygen consumption was measured by using a Clark electrode in isolated rat liver mitochondria. Paclitaxel up to the concentration of 20 μM either alone, or in combination with up to 500 μM Ca^{2+} did not decrease the oxygen consumption supported by either succinate, or pyruvate under our experimental conditions.

Effect of amiodarone and desethylamiodarone on the mitochondrial oxygen consumption. The oxygen consumption of isolated mitochondria (state 4 respiration) was measured by a Clark electrode, with 10 mM pyruvate (Complex I supported respiration) or 10 mM succinate in the presence of 1 μM rotenone (Complex II supported respiration) exposed to different concentrations of amiodarone or desethylamiodarone. In isolated rat heart mitochondria, at low concentrations, amiodarone did not have a significant effect either on Complex I or on Complex II supported respiration, as compared to the control. In an intermediate concentration range amiodarone increased both the Complex I- and Complex II-supported oxygen consumption indicating an uncoupling effect, while, the drug gradually inhibited respiration at higher concentrations.

Desethylamiodarone did not have any significant effect on the mitochondrial oxygen consumption at low concentrations of up to 10 μM. Above this concentration it gradually inhibited the respiration supported by succinate and in the concentration above 30 μM the

respiration supported by pyruvate, without presenting an uncoupling effect, as indicated by the absence of the stimulation of both Complex I- and Complex II-supported respiration in the concentration range of 6 to 30 μM. Above 30 μM, desethylamiodarone inhibited Complex I supported respiration, similarly to that observed with equimolar concentrations of amiodarone. However, above the concentration of 30 μM, desethylamiodarone presented a significantly stronger inhibition ($p < 0.01$) on Complex II supported respiration, than the one observed with equimolar concentrations of amiodarone. The effect of amiodarone or desethylamiodarone on isolated rat liver mitochondria was basically the same.

Conclusions.

1. Taxol directly affects the mitochondria by inducing a concentration dependent CsA-sensitive permeability transition, and cyt-c release, whereas Ad and Dea induced a slow rate concentration dependent swelling above the concentration of 10 μM that was not affected by CsA.

2. Both Ad and taxol caused the dissipation of ΔΨ in a concentration dependent manner. The dissipation of ΔΨ caused by taxol was CsA sensitive, whereas the dissipation of ΔΨ induced by Ad was not affected by CsA. These data indicate that taxol induces MPT through the ANT. Ad induces the swelling by a different mechanism independent of CsA. Dea does not cause the dissipation of ΔΨ in low concentrations.

3. Ad delayed the onset of calcium induced swelling up to the concentration of 10 μM, whereas Dea did not inhibit swelling in low concentrations.

4. Ad and Dea did not induce ROS production. Taxol induced ROS-formation in isolated liver mitochondria in a concentration dependent manner, which was localized mainly in the mitochondrial membrane.

5. Taxol, apart from the previously described mechanisms, also causes apoptosis through a direct mitochondrial pathway by inducing IM permeabilization and cytochrome c release through ANT, and stimulating ROS production through cytochrome oxidase, which may also contribute to MPT.

6. Ad exerts a biphasic effect on the mitochondria, with protective effects in lower concentration and toxic properties manifesting, when present at higher concentrations, whereas Dea does not have this dual feature.

7. Ad but not Dea protects the mitochondrial energy metabolism of the perfused heart during ischemia and reperfusion. This protective effect is presumably due to the inhibitory effect of Ad on the mitochondrial permeability transition.

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