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The effect of aging on mitochondrial and cytosolic hepatic intrinsic death pathway and apoptosis associated proteins in Fischer 344 rats

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ABSTRACT

Apoptosis is increased in the liver in old age and is a common pathological feature of liver disease. The mitochondria play a key role in regulating apoptosis via the intrinsic death pathway. As the effect of aging on this pathway is unclear, we aimed to characterize the impact of aging on the hepatic intrinsic death pathway and apoptosis. Livers from young adult (6.6 ± 0.3 months, n = 9) and old (25.4 ± 0.7 months, n = 9) male Fischer 344 rats were extracted for cellular fractionation and immunobloting. In old age there were lower mitochondrial protein levels of pro-apoptotic BAK, BID, tBID and VDAC1 ($p < 0.05$) and of anti-apoptotic Bcl-2. Compared to young, old rats had lower cytosolic protein levels of pro-apoptotic BAX, BAK, BID, tBID and anti-apoptotic Bcl-xL ($p < 0.05$). BAK, Bcl-2 and Bcl-xL were found in the cytosol. Furthermore with old age, cytosolic protein levels of cytochrome C, AIF and cleaved caspase-9 did not change but activation of caspase-3, -6 and -7 increased ($p < 0.05$) and DNA fragmentation trended to increase. Our results suggest an age-related decline in the levels of a number of proteins involved in the intrinsic death pathway, an uncoupling of intermediate apoptosis signaling and increased cellular apoptosis in the liver in old age.

1. Introduction

Aging is characterized by increased hepatic apoptosis (Molpeceres et al., 2007; Muskhelishvili et al., 1995; Zhang et al., 2002). Apoptosis is a tightly regulated and coordinated process of cell collapse which prevents the release of harmful substances in un-programmed cell death. This process is characterized by membrane blebbing, cell shrinkage, condensation of chromatin and fragmentation of DNA (Kam and Ferch, 2000). In addition to its important role in development, tissue remodeling and removal of damaged cellular debris, apoptosis is a protective mechanism that reduces inflammation during cellular insults such as drug toxicity (Kam and Ferch, 2000). Modification of this mechanism results in detrimental outcomes such as tumor formation, liver disease and degenerative diseases (Hanahan and Weinberg, 2011; Honig and Rosenberg, 2000; Wang, 2014).

Mitochondria have been shown to play a crucial role in the decision phase of cell death and to regulate apoptosis through the intrinsic death pathway (Kam and Ferch, 2000; Pollack and Leeuwenburgh, 2001). Following death stimulus, mitochondrial outer membrane permeabilization (MOMP) occurs as a result of translocation of certain proteins in the B-cell lymphoma-2 (Bcl-2) protein family from the cytosol to the mitochondrial outer membrane (Youle and Strasser, 2008). This family consists of three groups of proteins which include (a) pro-apoptotic proteins (Bcl-2-associated X protein (BAX), Bcl-2-antagonist or killer (BAK)), (b) BH-3 only proteins which modulate apoptotic proteins (Bcl-2 homology 3-interacting domain death agonist (BID)) and (c) anti-apoptotic proteins (B cell lymphoma-2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL)) (El-Hassan et al., 2003; Monaghan et al., 1992; Youle and Strasser, 2008). BAX and BID have been shown to reside in the cytosol and translocate to the mitochondria. Although Bcl-xL is

Abbreviations: BAX, Bcl-2-associated X protein; BAK, Bcl-2-antagonist or killer; BID, Bcl-2 homology 3-interacting domain death agonist; Bcl-2, B cell lymphoma-2; Bcl-xL, B-cell lymphoma-extra large; VDAC-1, Voltage Dependent Anion Channel 1 porin; MOMP, mitochondrial outer membrane permeabilization; AIF, Apoptosis inducing factor.

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mainly expressed in the mitochondrial membrane, it has also been found in the cytosol (El-Hassan et al., 2003; Kaufmann et al., 2003; Walensky and Gavathiotis, 2011). Bcl-2 and BAK are generally accepted to be expressed in the mitochondrial membrane (Lindsay et al., 2011), however recently BAK has also been found in the cytosol (Todt et al., 2015). Overall the pivotal balance in the mitochondrial outer membrane between opposing anti- and pro-apoptotic proteins determines the fate of the cell. In circumstances of increased pro-apoptotic stimulation, BAX, BAK, BID and truncated BID (tBID) proteins dimerize and form pores in the mitochondrial outer membrane resulting in MOMP, while anti-apoptotic proteins prevent pore formation (Peixoto et al., 2011; Wei et al., 2001; Youle and Strasser, 2008). BAX, BAK and BID have also been shown to activate and open the megapore, Voltage Dependent Anion Channel 1 porin (VDAC1), which is believed to escalate MOMP (Shimizu et al., 1999). Permeabilization of the mitochondrial outer membrane results in the release of the intermediate apoptosis signaling molecule cytochrome c causing activation of caspase-9, which then cleaves and activates caspase-3, followed by cleavage of caspase-6 and -7 resulting in downstream apoptosis and DNA fragmentation (Cullen and Martin, 2009; Elmore, 2007; Tait and Green, 2010; Zhang et al., 2002). MOMP also causes leakage of Apoptosis Inducing Factor (AIF) which translocates from the mitochondria to the nucleus causing nuclear condensation and apoptosis that is independent of caspase activity (Cande et al., 2004).

The mammalian liver has a multitude of crucial physiological roles (i.e. carbohydrate metabolism, plasma protein synthesis, lipid metabolism (Miller et al., 1951; Nguyen et al., 2008; Raddatz and Ramadori, 2007) and is vital for drug clearance and detoxification. A common finding in the aging liver is increased apoptosis which may contribute to the age-related increases in the incidence of liver disease (Frith et al., 2009), non-alcoholic fatty liver disease (Lee et al., 2007), adverse drug effects and changes in drug clearance (Hilmer et al., 2005), but this is poorly understood. Interestingly, apoptosis has also been commonly observed in the pathology of liver disease caused by drugs and other stressors (Wang, 2014). The impact of the age associated increase in hepatic apoptosis on the ability of the liver to counteract stressors is unclear.

Currently there is limited knowledge about the age-related changes in the regulation of hepatic mitochondrial dependent apoptosis. In Wistar rats Molpeceres et al. (2007) showed age-related changes in the intrinsic death pathway that included increased BAX, decreased Bcl-2 and increased cytochrome c leakage from liver mitochondria. However there are other proteins that are also involved in the complex process of programmed cell death that need investigation. Recent data suggest the key roles of BAX, BAK and/or VDAC-1 in MOMP (Keinan et al., 2010; Wei et al., 2001). In addition, modulation of BID and Bcl-xL can alter the degree of programmed cell death (Takehara et al., 2004; Yin et al., 1999). The effect of aging on the mitochondrial expression levels of other proteins involved in the mitochondrial intrinsic death pathway, such as BAK, VDAC-1, BID and Bcl-xL, has not been characterized. In addition, little is known about the effect of aging on the cytosolic levels of these proteins.

Therefore this study was undertaken to characterize in young and old Fischer 344 rats, protein levels of the hepatic mitochondrial and cytosolic intrinsic death pathway and apoptosis associated proteins. Fischer 344 rats are a commonly used aging model with minimal age associated obesity relative to other species of rats (Schneider, 2012), which has been shown to affect apoptosis (Ferreira et al., 2011).

2. Experimental procedures

2.1. Animals

Young adult (6.6 ± 0.3 months, 381.2 ± 9.4 g, n = 9) and old (25.4 ± 0.7 months, 424.8 ± 14.5 g, n = 9) male Fischer 344 rats were obtained from Harlan Laboratories (Harlan, Bethesda, Maryland). Animals had access to water and food ad libitum (Rat and Mouse Breeder Diet; Gordon's Specialty Stockfeeds, Yanderra, NSW, Australia). The study was approved by the Royal North Shore Hospital Animal Care and Ethics Committee and the University of Sydney Animal Ethics Committee.

2.2. Materials

Acetaminophen, Igepal and Protease inhibitor cocktail were purchased from Sigma-Aldrich (Sydney, Australia).

2.3. Treatment of animals and subsequent tissue collection

As part of a larger study we have described previously (Mach et al., 2013), animals were fasted overnight for 18 h before intraperitoneal injection (*i.p.*) of saline between 10 am and 12 pm. Four hours post saline injection animals were anesthetized with *i.p.* injection of Ketamine (75 mg/kg; Parnell Laboratories Pty Ltd, Sydney, Australia) and Xylazine (10 mg/kg; Troy Laboratories Pty Ltd, Sydney, Australia).

After performing a midline laparotomy, blood was taken from the inferior vena cava followed by an injection of heparin (300 U) into the same vessel. The portal vein was then cannulated with an 18G intravenous catheter (BD, Sydney, Australia) and the liver was perfused in-situ with oxygenated Krebs–Henseleit Bicarbonate buffer (95% O_2–5% CO_2, 37 °C) at a flow rate of 1–1.5 mL/min/g of liver to remove blood from the liver tissue. The liver was then excised and segments were placed into Cryovials (Proscitech, Brisbane, Australia) and snap frozen in liquid nitrogen for subsequent immunoblotting. A section of the main lobe was also fixed in 10% neutral buffered formalin (Proscitech, Brisbane, Australia) for histology.

2.4. Histology and serum biochemistry

Fixed liver tissue was embedded in paraffin and 5 μm sections were mounted on slides. Slides were processed for hematoxylin and eosin staining in a National Association of Testing Authorities accredited hospital laboratory of the Pathology department of Royal Prince Alfred Hospital, Sydney, Australia. Histopathology was described by an anatomical pathologist (CM), who was blinded to the age group of the samples. Animals with histopathologic evidence of age-related hepatic malignancy, lymphoproliferative disease or other liver diseases were excluded (n = 0/ 9 young and n = 4/ 13 old).

The collected blood was allowed to clot at room temperature before separating the serum by centrifugation at 10,000 RPM (7378 g) for 10 min (Sigma centrifuge 1–14, John Morris Scientific Pty Ltd, Australia). Sera were then stored in aliquots at −80 °C. Serum liver function tests consisting of alanine transaminase activity and aspartate transaminase activity were carried out to assess liver injury. These tests were conducted by a National Association of Testing Authorities accredited hospital laboratory, PaLMS (Pacific Laboratory Medicine Services) (PaLMS, 2013) at Royal North Shore Hospital (Sydney, Australia).

2.5. Western analysis

Liver tissue was homogenized with a dounce homogenizer (Sigma-Aldrich, Sydney, Australia) and the cells were fractionated into cytosolic, mitochondrial and nuclear fractions using the method adapted from Ishigami et al. (2001) and Kislinger et al. (2003). In brief, samples were homogenized in Buffer 1 (250 mM sucrose, 10 mM KCl, 20 mM HEPES-KOH (pH 7.4), 1.5 mM MgCl, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM DTT, 1 mM PMSF, 10 μl/ml Protease inhibitor cocktail) followed by centrifugation at 800 *g* for 10 min at 4 °C (Sigma centrifuge 3-16pk, John Morris Scientific Pty Ltd, Australia). The pellet was washed two times with Buffer 1 and resuspended in Buffer 2 (0.42 M NaCl, 25% glycerol, 20 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 μl/ml Protease inhibitor cocktail). This was followed by repeated suction and