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The intrinsic apoptotic pathway lies upstream of oxidative stress in multiple organs

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Abstract

Oxidative stress increases with age in multiple organ systems and is implicated in the development of age-related pathologies in them. Studies from our laboratory show that the intrinsic pathway proapoptotic proteins BAX and caspase-3 (CASP3) lie upstream of mitochondrial production of oxidative stress-inducing reactive species (RS) such as reactive oxygen and reactive nitrogen species (ROS and RNS) in apoptotic and nonapoptotic neurons in cell culture. Our objective in this study was to determine if these findings could be generalized to the development of oxidative stress in nonneuronal tissues *in vivo*. We first investigated the effect of genetic deletion of *Bax* on DNA damage in the liver, heart and kidneys of female mice of increasing ages (5, 14, 22 months). The organs of the aged mice showed increased oxidative DNA strand breaks compared to young animals (5 month). Ablation of *Bax* greatly reduced this damage. We next assessed lipid peroxidation, DNA oxidation, and protein tyrosine nitration to determine whether *Casp3* deletion reduces oxidative stress in the hearts, livers, and kidneys of 12-month-old female mice. Lipid peroxides and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were much lower in organs from mice with depleted *Casp3* than in those of wild type animals. Nitration of protein tyrosine residues, caused by RNS, was also significantly suppressed in the tissues of *Casp3* null mice compared to those in wild type mice. Our findings indicate that BAX and CASP3 have a vital role in the generation of oxidative stress in organs of aged mice.

Graphical Abstract

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Keywords

Apoptosis; Reactive Oxygen; Oxidative Stress; BAX; Caspase 3

1. Introduction

Increased levels of oxidative stress occur in many organ systems in aging humans and other animals. Evidence indicates that this stress contributes to multiple age-related pathologies including, but not limited to, Alzheimer's disease, stroke, cardiac disease, arteriosclerosis, kidney disease, and liver disease [1–13]. Oxidative stress may also contribute to the deleterious changes occurring in many tissues during the normal aging process [14]. The primary source of this stress is reactive chemical species (RS) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [15] that are derived from the electron transport chain of mitochondria or from the activity of certain enzymes [13, 16]. Cellular RS levels are regulated by a balance between their production and removal by antioxidants. RS have some normal physiological functions including roles in enzyme-catalyzed reactions, signal transduction, gene expression, and several other biological processes [17]. However, increased generation of RS and/or reduced antioxidant capacity can give rise to oxidative stress and subsequently damages proteins, lipids and DNA.

BAX is a pro-apoptotic protein involved in the intrinsic apoptotic pathway in a wide variety of tissues, including the brain, heart, liver, and kidney [18–21]. Insertion of BAX into the outer mitochondrial membrane triggers the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol where it induces formation of the apoptosome and subsequent activation of executioner caspases such as caspase-3 (CASP3) [22]. A large increase in RS levels occurs during apoptotic death of multiple cell types [23–27]. Previous

studies from our laboratory have shown that BAX and CASP3 lie upstream of increased production of mitochondria-derived superoxide ($O_2^{\bullet-}$) and RS lying downstream of $O_2^{\bullet-}$ in both apoptotic and nonapoptotic neurons in vitro and, thus, likely contributes to oxidative stress in the nervous system [23–27]. Effects on other cell types or organs is unknown. While investigating the role of BAX and CASP3 in generating oxidative stress in the brain, we also investigated their role in development of oxidative stress in several other organs. We report here on the role of BAX and CASP3 in development of oxidative stress in three of those organs, hearts, livers and kidneys. We found that deletion of *Bax* (*Bax*^{-/-}) and *Casp3* (*Casp3*^{-/-}) caused a decrease in oxidative damage to the hearts, livers, and kidneys of aged female mice. We first investigated the effect of BAX deletion on oxidative DNA damage in young (5 months), middle-aged (14 months), and old (22 months) mice. Removal of *Bax* greatly reduced oxidative damage at all ages. Next, we investigated whether genetic ablation of *Casp3* decreased oxidative stress markers for lipid peroxidation, oxidative DNA damage, and protein tyrosine nitration in the hearts, livers, and kidneys of twelve-month-old female mice. These markers were all prominently reduced in *Casp3*^{-/-} mice as compared to wild type mice (*Casp3*^{+/+}) of the same age. Our findings indicate that BAX and CASP3 lie upstream of most or all age-related increases in oxidative stress in the mouse liver, heart, and kidney.

2. Materials and methods

2.1. Reagents and antibodies

The ELISA kit for assessing oxidative DNA damage (Catalog # 589320) was obtained from Cayman Chemical (Ann Arbor, MI, USA). An antibody to 3-Nitrotyrosine (Catalog # ab61392) was purchased from Abcam (Cambridge, MA, USA). Unless otherwise mentioned, all other reagents were obtained either from Sigma-Aldrich (St. Louis, MO, USA) or (Pierce/Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Mice mating and genotyping

All animal procedures were reviewed and approved by the Animal Studies Committee at the University of Georgia. Under the supervision of two ACLAM certified veterinarians in an AAALAC-approved facility, all mice were placed on a 12 h light/dark cycle and given access to food and water *ad libitum*.

Arrested spermatogenesis causes male *Bax*^{-/-} mice to be infertile. Young *Bax*^{-/-} females are poor breeders [39]. *Sod2* knockout (*Sod2*^{-/-}) [29–31, 40, 41] mice die in the first week of life. Therefore, *Bax* and *Sod2* hemizygous mice (*Bax*^{+/-} *Sod2*^{+/-}; The Jackson Laboratory; Bar Harbor, ME, USA) were mated giving the following 9 genotypes: *Bax*^{+/+} *Sod2*^{+/+}, *Bax*^{+/+} *Sod2*^{+/-}, *Bax*^{+/+} *Sod2*^{-/-}, *Bax*^{+/-} *Sod2*^{+/+}, *Bax*^{+/-} *Sod2*^{+/-}, *Bax*^{+/-} *Sod2*^{-/-}, *Bax*^{-/-} *Sod2*^{+/+}, *Bax*^{-/-} *Sod2*^{+/-}, and *Bax*^{-/-} *Sod2*^{-/-}. Of these offspring, three different genetic combinations (*Bax*^{+/+} *Sod2*^{+/+}, *Bax*^{+/+} *Sod2*^{+/-}, and *Bax*^{-/-} *Sod2*^{+/-}) were chosen for this study. Ages of the mice chosen were based on their relevance to human aging and were 5-month-old “young” animals, 14-month-old “middle-aged” animals, and 22-month-old “old” animals [10]. Because of gender differences in oxidative stress levels in mice we chose to study only one sex, females, to reduce the number of animals needed [40].

Casp3 hemizygous mice (*Casp3*^{+/-}; The Jackson Laboratory) were mated resulting in the following three offspring: *Casp3*^{+/+}, *Casp3*^{+/-}, and *Casp3*^{-/-} (C57BL/6 genetic background). We chose 12-month-old female wild type (*Casp3*^{+/+}) and *Casp3* deleted (*Casp3*^{-/-}) mice for our study. The age selected represents middle-aged animals and was based on our *Bax* data showing that female middle-aged wild-type mice had somewhat higher oxidative stress levels than were found in young wild-type female mice and similar levels to those in old wild-type female mice (Fig. 1a–c). Genomic DNA was extracted from a tail snip of each mouse using Wizard Prep kits (Promega, Fitchburg, WI) or QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI, USA) or. The *Bax*, *Sod2*, or *Casp3* genotype of each mouse was determined by PCR.

2.3. Polymerase chain reaction (PCR) and verification of genotype

PCR was carried out using a Techne Genius PCR thermocycler (Burlington, NJ). For the genotyping of *Bax* and *Sod2*, 24 µl of reaction mixture (9 µl ddH₂O, 2.5 µl primers, 12.5 µl 2X MangoMix solution (Bioline, London, UK) and 1.5 µl of extracted DNA were combined in a 0.5 ml microcentrifuge tube. The PCR for the *Bax* gene was performed as previously described [25]. The primers used in the identification of the *Bax* gene were: intron reverse 5'-GTT GAC CAG AGT GGC GTA GG-3' and exon forward 5'TGA TCA GAA CCA TCA TG-3' to amplify a 304 pb sequence of the wild type allele; neo/pgk reverse 5'-CCG CTT CCA TTG CTC AGC GG-3' and same exon forward to amplify a 507bp sequence of the mutant allele. Cycling conditions were 33 cycles of 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min. The primers used for *Sod2* were: 5'GGC CTA CGT GAA CAA CCT CAA C-3' and 5'GAA AGG ACG TTT ATG CGA ACC AAC-3' to amplify a 134 bp sequence of the wild type allele; 5'CCA GTC TCA GGG GCA ACA AAG ATG-3' and 5'CGC CTA CCG GTG GAT GTG GAA TGT-3' to amplify a 311 bp sequence of the mutant allele. Cycling conditions were 35 cycles of 94° C for 30 sec, 59° C for 30 sec, and 72° C for 30 sec. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide using Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software (Fotodyne Inc., New Berlin, WI, USA). In addition to PCR, BAX and SOD2 protein levels in the different genotypes was verified by immunoblotting of neural tissue taken from the same animals as those used for determining oxidative stress in heart, liver, and kidneys (Supplementary Materials 1, 2).

PCR for *Casp3* was carried out as per the directions given by The Jackson Laboratory. Twenty-three µl of a reaction mixture (8.61 µl ddH₂O, 0.63 µl of each primer, and 12.5 µl 2X MangoMix solution) was mixed with 2 µl of extracted DNA. Primers used in this reaction were: wild type forward 5'-GCG AGT GAG AAT GTG CAT AAA TTC-3' and wild type reverse 5'-GGG AAA CCA ACA GTA GTC AGT CCT-3' for amplification of a 320 bp sequence of the wild type allele; neo/pgk forward 5'-TGC TAA AGC GCA TGC TCC AGA CTG-3' and wild type exon reverse to amplify a 300 bp sequence of the mutant allele. Thermocycling sequences for the PCR mixture were 94° C for 2 min followed by 10 cycles of 94° C for 20 sec, 65° C for 15 sec, and 68° C for 10 sec. This was followed by 28 cycles of 94° C for 15 sec, 60° C for 15 sec, and 72° C for 10 sec. PCR products were separated by 3% (w/v) agarose gel electrophoresis and analyzed with a Fotodyne Foto/Analyst Dual-

Light Luminary Workstation with TotalLab software. DNA base pairs were visualized with the help of ethidium bromide present in the agarose gel.

2.4. Thiobarbituric acid reactive substances (TBARS) assay

Oxidative stress caused by increased production of ROS can modify membrane lipids into highly reactive and unstable lipid peroxides that eventually break down into more stable products like malondialdehyde (MDA). This can be estimated by the TBARS assay, a technique based on the reaction of MDA with thiobarbituric acid (TBA) [42]. Frozen tissues were thawed on ice and then homogenized in a 10% w/v KCl, and centrifuged at 800x g for 10 min at 4° C. The supernatant obtained was used for measuring the protein concentration using the Bradford Assay. For the TBARS assay, 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, 10M sodium hydroxide (for pH 3.5) and 0.8% TBA was added to the appropriate amount of supernatant. This mixture was then diluted to 1 ml with dH₂O and placed in a water bath at 100° C for 1 h. After cooling, 1 ml of dH₂O and 1 ml of butanol/pyridine (15:1, v/v) mixture was added to this reaction mixture and shaken gently. From this, a 200 µl aliquot was pipetted out in a 96-well plate and the levels of MDA were detected by measuring the absorbance at 532 nm using a Molecular Devices SpectraMax M2 spectrophotometer (Molecular devices, San Jose, CA, USA). Each sample was performed in triplicate. The concentration of lipid peroxides in the sample was calculated from a MDA standard curve that was prepared using 0-10 µM tetraethoxypropane, and was expressed as µM MDA per mg protein.

2.5. Analysis of oxidative DNA damage

Oxidative DNA damage was first assessed in liver, heart, and kidney tissues using single cell gel electrophoresis (comet assay) [32,33]. 25mg of tissues that had been stored at -80 C° was thawed and dissociated in ice-cold PBS (1 ml). The resulting cell suspension was embedded in 1% (w/v) low melting point agarose and placed on agarose-coated glass slides. After the gels set, the slides were immersed in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM EDTA, 10mM Trizma base, pH 10) for 1 h. They were then subjected to electrophoresis at 25mV (400mA) for 25 min. After drying overnight, slides were visualized by staining with 20 mM ethidium bromide for 30 min. Images were acquired using fluorescence microscopy and MetaMorph software (Molecular Devices, San Jose, CA, USA). TriTek CometScore™ (TriTek, Sumerduck, VA, USA) software scored the comets. Results are reported as %DNA in tail, normalized to young wild type *Bax*^{+/+}/*Sod2*^{+/+} animals.

Oxidative damage to DNA causes changes in purine and pyrimidine bases, single and double-stranded breaks, and cross-linking with other biomolecules [43, 44]. Among these bases, guanine is highly sensitive to oxidation. Oxidative damage to DNA was quantified using a DNA/RNA oxidative damage ELISA kit that measures all three oxidized guanine species; 8-hydroxy-2'-deoxyguanosine (8-OHdG) from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. DNA extracted from the heart, liver, and kidney of wild type (*Casp3*^{+/+}) and caspase-3 mutant (*Casp3*^{-/-}) was added to wells of ELISA plates previously treated with a goat polyclonal anti-mouse IgG. A DNA/RNA oxidative-damage monoclonal antibody that forms a complex with the oxidatively damaged

guanine within DNA, was added. This complex then binds with the attached anti-mouse IgG. An enzymatic reaction takes place on addition of Ellman's Reagent to each well, resulting into a yellow colored product whose absorbance was measured at 410 nm using a Molecular Devices SpectraMax M2 spectrophotometer. Analysis of each sample was done in duplicate. The amount of oxidized DNA was quantified using a standard curve and expressed as pg/ml.

2.6. Immunoblot analysis

The mouse tissues were thawed and homogenized with a glass dounce homogenizer in ice-cold RIPA lysis buffer containing a protease inhibitor cocktail (Calbiochem #539134, La Jolla, CA, USA). Homogenates were then centrifuged for 10 min at 10000x g. The supernatant was used for determining protein concentration using the Bradford assay ((Pierce/Thermo Fisher). Each sample containing 50 µg protein and sample buffer (100 mM Tris-HCl, 4% SDS, 10% bromophenol blue, 30% glycerol) was loaded into individual wells of 12% Tris-HCl pre-cast gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A protein molecular weight marker was added in one of the lanes. After loading, SDS-PAGE (Polyacrylamide gel electrophoresis) was carried out by running the gel at 100V for 1 h. Proteins were then transferred onto PVDF membranes (Millipore, Bedford, MA, USA) at 100 V for 1-2 h. This was followed by incubating the membrane with blocking buffer consisting of 1% bovine serum albumin in TBS-T (10 mM Tris-HCL, 100 mM NaCl, and 0.1% Tween-20) for 30 min at room temperature with gentle shaking. Mouse anti-3-Nitrotyrosine (3-NT) monoclonal antibody (1:1000 dilution) was then added into the blocking buffer and incubated the membrane for overnight at 4° C with gentle shaking. After overnight incubation, the membrane was washed 3X with the wash buffer (TBS-T), with shaking. Each rinse was for 5-10 min. After incubating the membrane with anti-mouse IgG, HRP-linked secondary antibody (1:2000 dilution, Cell Signaling, Danvers, MA, USA, Catalog# 7076) for 1 h at room temperature with gentle shaking, the washes with TBS-T were repeated. Proteins were detected after incubating the membrane in Chemiluminescent ECL Western Blotting Substrate (Pierce/Thermo Fisher, Waltham, MA, USA). Visualization of immunoblots and their analyses was done using a Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software. The protein band density representing 3-Nitrotyrosine for each sample was normalized to the respective β -actin levels, and the data was represented as relative intensity to β -actin. Primary antibody to β -actin (Sigma-Aldrich, catalog# A2228) was used at 1:1000 dilution and a secondary anti- mouse HRP-linked IgG (Cell Signaling, Catalog# 7076) at 1:5000 dilution.

2.7. Statistical analysis

Graphical representations and statistical analysis were performed using SigmaPlot 11.1 (Systat Software, Inc., San Jose, CA, USA). Analysis of data distributions was used to determine appropriate statistical measures. Statistical comparisons of multiple groups were made via Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's Multiple Comparison Test. Statistical comparisons of two groups was done either by *t*-test or Mann-Whitney *U*-test depending on the data distribution. All error bars are $\pm$ SEM.

3. Results

3.1. Genetic deletion of Bax decreased oxidative stress in the aging mouse liver, heart, and kidney

Our previous work shows that Bax lies upstream of most RS produced in sympathetic and cerebellar granule neurons in cell culture [27]. As an adjunct to experiments aimed at investigating the role of BAX in cortical neurons in cell culture and oxidative stress in the brain, we screened for a role for BAX in development of oxidative stress in three other organs. To explore whether the intrinsic apoptotic pathway influences the development of oxidative stress with increasing age, we first determined the effect of *Bax* deletion on development of oxidative stress-induced DNA damage in liver, heart, and kidney in female mice aged 5, 14, and 22 months. Most tissues, including these organs, express BAX [28]. Superoxide dismutase 2 (SOD2) is an antioxidant that mitigates RS levels in healthy tissues. The genotypes we chose to study were wild type (*Bax*^{+/+}/*Sod2*^{+/+}), those under chronic oxidative stress caused by reduced levels of SOD2 (*Bax*^{+/+}/*Sod2*^{+/-}) [29–31], and those with reduced SOD2 levels but lacking BAX (*Bax*^{-/-}/*Sod2*^{+/-}). Single-cell gel electrophoresis, known as the comet assay, is widely used in the determination of oxidative and other types of DNA damage (double and single strand breaks; [32, 33]). Undamaged DNA exists as a tightly coiled package in the cellular nucleus. If damaged, this organization becomes more relaxed, allowing breaks in the DNA strands to expand outside of the nucleus. The application of an electric field to agarose-coated glass slides containing cellular nuclei drives the migration of the damaged DNA fragments in proportion to the amount of damage in the cell, forming a comet-shaped “tail”. Undamaged DNA strands are too large to migrate out of the comet “head” and thus remain in a compact circular shape. The tail can then be analyzed using software specific for comet parameters.

Using the comet assay, we found that there were significant increases in DNA strand breaks in wild type kidney and in both heart and kidney of *Bax*^{+/+}/*Sod2*^{+/-} mice by 14 months of age compared to the levels at 5 months (Fig. 1a–c). There was no significant increase in liver DNA strand breaks in either genotype at any age. Strand breaks remained elevated at 22 months in *Bax*^{+/+}/*Sod2*^{+/-} heart but had decreased to 5-month-old levels in *Bax*^{+/+}/*Sod2*^{+/-} kidneys at this time. *Bax*^{+/+}/*Sod2*^{+/+} strand breaks remained elevated only in kidneys at 22 months of age. Livers of *Bax*^{+/+}/*Sod2*^{+/+} animals did not show significantly different strand breaks at any age. Strand breaks were higher at some ages in *Bax*^{+/+}/*Sod2*^{+/-} than in wild type in all three tissues, consistent with increased oxidative stress in those tissues due to reduced SOD2 levels. DNA strand breaks were significantly inhibited in all three tissues from mice aged 14 and 22 months by deletion of *Bax* (Fig. 1a–c).

3.2. Genetic deletion of Casp3 decreased oxidative damage in the aged mouse liver, heart, and kidney

After establishing a possible role for BAX in development of age-related oxidative stress in the three organs with the TBARS and comet assays, we next investigated its main downstream effector, CASP3. To determine the effects of *Casp3* deletion on oxidative stress in the three tissues, we assessed the levels of lipid peroxides with the TBARS assay, oxidized DNA by 8-OHdG ELISA, and protein tyrosine nitration with Immunoblotting. We

chose to use the 8-OHdG assay in these experiments because it is a more definitive measure of oxidative DNA damage than is the comet assay which indicates DNA damage that may be caused by RS but could also have other causes. Determination of 3-Nitrotyrosine levels provides a marker of protein oxidation. We chose 12-month-old female mice (C57Bl/6 genetic background) for these experiments as this age was near the peak of DNA damage as shown in figure 1 and others have shown an increase in oxidative stress in mice of this age [34]. Nitration of protein tyrosine residues caused by RNS such as peroxynitrite (ONOO^-) was much higher in the livers of wild type mice than in *Casp3* ablated animals (Fig. 2a). DNA oxidation, as assessed by measuring the levels of 8-OHdG, in liver was also greatly inhibited by deletion of *Casp3* (Fig. 2b). The levels of MDA, a lipid peroxidation product detected by the TBARS assay, were significantly reduced in the liver tissue of *Casp3*^{-/-} mice in comparison to livers of wild type (*Casp3*^{+/+}) mice (Fig. 2c).

As in the liver, *Casp3* deletion greatly decreased 3-Nitrotyrosine levels in aged mouse heart, suggesting a vital role for CASP3 protein in producing oxidative stress in cardiac tissue (Fig. 3a). Oxidative DNA damage in 12-month-old mouse hearts was also greatly reduced in the *Casp3*^{-/-} mice (Fig. 3b) as were MDA levels (Fig. 3c). Figure 4a shows a large decrease in the 3-Nitrotyrosine content of proteins in the kidney of *Casp3*^{-/-} mice in comparison to *Casp3*^{+/+} mice. Oxidation of DNA was also prominently reduced in the kidneys from these mice (Fig. 4b). *Casp3*^{-/-} mice also had greatly decreased levels of lipid peroxides in comparison to *Casp3*^{+/+} mice (Fig. 4c). Taken together, our results indicate that genetic deletion of *Casp3* profoundly suppresses oxidative stress in multiple organs of middle-aged female mice.

4. Discussion

Many tissues in older animals have elevated levels of oxidative stress as compared to similar tissues in younger animals. This increased stress contributes to many age-related pathologies and, possibly, the normal aging process [1–14]. While an imbalance between RS production and removal by antioxidants likely underlies age-related increases in oxidative stress, the causes of this imbalance remain unclear [13, 17]. This study was aimed at elucidating the role of the intrinsic apoptotic pathway this imbalance. Our previous studies indicate that BAX and CASP3, major molecular components of the intrinsic apoptotic pathway in many cell types, lie upstream of most apoptotic and nonapoptotic RS production in neurons [23–27]. Based on these findings, we hypothesized that BAX and CASP3 might also contribute to oxidative stress in nonneuronal tissues. We chose to explore this possibility in three organs that exhibit increased oxidative stress with age, liver, heart, and kidney, and that express BAX and CASP3 [28].

We first assessed oxidation of DNA in *Bax*^{+/+} and *Bax*^{-/-} female mice of young, middle, and old ages. Overall, oxidative DNA damage increased with age in the organs of the *Bax*^{+/+} mice but not in the *Bax*^{-/-} mice. We next assessed the role of CASP3 in DNA, Lipid, and protein oxidation in livers, hearts, and kidneys of twelve-month old *Casp3*^{+/+} and *Casp3*^{-/-} female mice by measuring three relevant oxidative stress markers. 8-OHdG, a measure of oxidative DNA damage, levels were far lower in all three organs from *Casp3*^{-/-} animals than in those from *Casp3*^{+/+} mice. 3-Nitrotyrosine, a marker for RNS damage to proteins, was

also greatly reduced in *Casp3*^{-/-} mice as was lipid peroxidation measured by the TBARs assay. Therefore, we conclude that both BAX and CASP3 lie upstream of oxidative stress in all three organs.

The pro-oxidant effect of BAX likely resulted from the dependence of CASP3 activation on BAX-induced release of cytochrome c from mitochondria. The CASP3 was then responsible for causing the increased oxidative stress. We did not explore the exact means by which this occurred. One known mechanism by which CASP3 can increase mitochondrial ROS production is by cleaving the p75 subunit (Ndufs1, NADH-ubiquinone oxidoreductase subunit) of respiratory complex I. This cleavage causes disruption of the electron transport chain and increased leakage of electrons to O₂ to cause its reduction to O₂^{•-} [35–37]. Another mechanism that increases production of mitochondrial ROS is through disruption of the interaction between the cytochrome c1 subunit of complex III and cytochrome c. This disruption is mediated by CASP3 through cleavage of cytochrome c1 at the D106 site that is essential for binding with cytochrome c, thus increasing O₂^{•-} production [38].

Large increases in ROS occur during the apoptotic death of many cell types [23–27]. Considering that BAX and CASP3 lie upstream of this stress in the three organs investigated it is reasonable to surmise that increased apoptosis accounts for the increased stress. However, our work with neurons demonstrates that apoptotic death is not required for BAX or CASP3 to have pro-oxidant effects [27]. Deletion of either *bax* or *Casp3* decreases basal ROS production in those cells. Low levels of basal (non-apoptotic) caspase activity exist in numerous cell types and is likely responsible for the pro-oxidant effect of BAX and CASP3 in non-apoptotic cells. Removal of BAX and CASP3 greatly reduced the oxidative damage to all three tissues examined in this study. We did not determine whether this might be accounted for by decreased apoptosis, might reflect effects on basal ROS production, or both.

In conclusion, our findings indicate that *Bax* or *Casp3* deletion greatly attenuates oxidative stress in the livers, hearts, and kidney of aged mice. These findings indicate that BAX and CASP3 lie upstream of generation of most or all age-related increases in oxidative stress in these three organ systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Butterfield DA, Perluigi M, Sultana R, Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics, *Eur. J. Pharmacol* (2006) 545 39–50. [PubMed: 16860790]
- [2]. Floyd RA, Hensley K, Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases, *Neurobiol. Aging* 23 (2002) 795–807. [PubMed: 12392783]

- [3]. Alexandrova ML, Bochev PG, Oxidative stress in the chronic phase after stroke, *Free Radic. Biol. Med* 39 (2005) 297–316. [PubMed: 15993329]
- [4]. Abramov AY, Scorziello A, Duchen MR, Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation, *J. Neurosci* 27 (2007) 1129–1138. [PubMed: 17267568]
- [5]. Abete P, Napoli C, Santoro G, Ferrara N, Tritto I, Chiariello M, Rengo F, Ambrosio G, Age-related decrease in cardiac tolerance to oxidative stress, *J. Mol. Cell Cardiol.* 31 (1999) 227–236. [PubMed: 10072730]
- [6]. Miró Ó, Casademont J, Casals E, Perea M, Urbano-Márquez Á, Rustin P, Cardellach F, Aging is associated with increased lipid peroxidation in human hearts, but not with mitochondrial respiratory chain enzyme defects, *Cardiovasc. Res* 47 (2000) 24–631.
- [7]. Brinkley TE, Nicklas BJ, Kanaya AM, Satterfield S, Lakatta EG, Simonsick EM, Sutton-Tyrrell K, Kritchevsky SB, Plasma oxidized low-density lipoprotein levels and arterial stiffness in older adults: the health, aging, and body composition study, *Hypertension* 53: (2009) 846–852. [PubMed: 19332658]
- [8]. Daenen K, Andries A, Mekahli D, Van Schepdael A, Jouret F, Bammens B, Oxidative stress in chronic kidney disease, *Pediatr. Nephrol* 34 (2019) 975–991. [PubMed: 30105414]
- [9]. Zhu R, Wang Y, Zhang L, Guo Q, Oxidative stress and liver disease, *Hepatol. Res* 42 (2012) 741–749. [PubMed: 22489668]
- [10]. Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A, Does oxidative damage to DNA increase with age, *Proc. Natl. Acad. Sci. USA* 98 (2001) 10469–10474. [PubMed: 11517304]
- [11]. Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, Gargiulo G, Testa G, Cacciatore F, Bonaduce D, Abete P, Oxidative stress, aging, and diseases, *Clin. Interv. Aging* 13 (2018) 757–772. [PubMed: 29731617]
- [12]. Wallace DC, A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine, *Ann. Rev. Gene* 39 (2005) 359–407.
- [13]. Halliwell B, Gutteridge JMC, *Free Radicals in Biology and Medicine*, Oxford University Press, New York, 2015.
- [14]. Gladyshev VN, On the cause of aging and control of life- span: heterogeneity leads to inevitable damage accumulation, causing aging; control of damage composition and rate of accumulation define lifespan, *Bioessays* 34 (2012) 925–929. [PubMed: 22915358]
- [15]. Radi R, Cassina A, Hodara R, Nitric oxide and peroxynitrite interactions with Mitochondria, *Biol. Chem* 383 (2002) 401–409. [PubMed: 12033431]
- [16]. Turrens JF, Superoxide production by the mitochondrial respiratory chain, *Biosci. Rep* 17 (1997) 3–8. [PubMed: 9171915]
- [17]. Hancock JT, Desikan R, Neill SJ, Role of reactive oxygen species in cell signalling Pathways, *Biochem. Soc. Trans* 29 (2001) 345–350. [PubMed: 11356180]
- [18]. Feldman G, Liver apoptosis *J Hepatol.* 26 (1997) 1–11.
- [19]. Hou Q, Hsu YT, Bax translocates from cytosol to mitochondria in cardiac cells during apoptosis: development of a GFP-Bax-stable H9c2 cell line for apoptosis analysis, *Am. J. Physiol. Heart Circ. Physiol.* 289 (2005) 477–487.
- [20]. Saikumar P, Venkatachalam MA, Role of apoptosis in hypoxic/ischemic damage in the kidney, *Semin. Nephrol* 23 (2003) 511–521. [PubMed: 14631559]
- [21]. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC, Immunohistochemical determination of in vivo distribution of BAX, a dominant inhibitor of Bcl-2, *Am. J. Pathol* 145 (1994) 1323–1336. [PubMed: 7992838]
- [22]. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X, Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, *Cell* 91 (1997) 479–489. [PubMed: 9390557]
- [23]. Tan S, Sagara Y, Liu Y, Maher P, Schubert D, The regulation of reactive oxygen species production during programmed cell death, *J. Cell Biol.* 141 (1998) 1423–1432. [PubMed: 9628898]

- [24]. Kirkland RA, Franklin JL, Evidence for redox regulation of cytochrome c release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition, *J. Neurosci* 21 (2001) 1949–1963. [PubMed: 11245680]
- [25]. Kirkland RA, Windelborn JA, Kasprzak JM, Franklin JL, A Bax-induced pro-oxidant state is critical for cytochrome c release during programmed neuronal death, *J. Neurosci* 22 (2002) 6480–6490. [PubMed: 12151527]
- [26]. Kirkland RA, Franklin JL, Bax affects production of reactive oxygen by the mitochondria of non-apoptotic neurons, *Exp. Neurol* 204 (2007) 458–461. [PubMed: 17097638]
- [27]. Kirkland RA, Saavedra GM, Cummings BS, Franklin JL, Bax regulates production of superoxide in both apoptotic and nonapoptotic neurons: role of caspases, *J. Neurosci* 30 (2010) 16114–16127. [PubMed: 21123558]
- [28]. Bouvard V, Zaitchouk T, Vacher M, Duthu A, Canivet M, Choisy-Rossi C, Nieruchalski M, May E, Tissue and cell-specific expression of the p53-target genes: bax, fas, mdm2 and waf1/p21, before and following ionising irradiation in mice, *Oncogene* 19 (2000) 649–660. [PubMed: 10698510]
- [29]. Van Remmen H, Salvador C, Yang H, Huang TT, Epstein CJ, Richardson A, Characterization of the antioxidant status of the heterozygous manganese superoxide dismutase knockout mouse, *Arch. Biochem. Biophys* 363 (1999) 91–97. [PubMed: 10049502]
- [30]. Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang TT, Nelson J, Strong R, Richardson A, Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging, *Physiol. Genomics* 16 (2003) 29–37. [PubMed: 14679299]
- [31]. Jang YC, Van Remmen H, The mitochondrial theory of aging: insight from transgenic and knockout mouse models, *Exp. Gerontol* 44 (2009) 256–260. [PubMed: 19171187]
- [32]. Fairbairn DW, Olive PL, O'Neill KL, The comet assay: a comprehensive review, *Mutat. Res* 339 (1995) 37–59. [PubMed: 7877644]
- [33]. Piperakis SM, Visvardis EE, Tassiou AM, Comet assay for nuclear DNA damage, *Methods Enzymol.* 300 (1999) 184–194. [PubMed: 9919521]
- [34]. Lim JH, Kim EN, Kim MY, Chung S, Shin SJ, Kim HW, Yang CW, Kim YS, Chang YS, Park CW, Choi BS, Age-associated molecular changes in the kidney in aged mice, *Oxidative Med. Cell. Longev.* 2012 (2012) 1–10.
- [35]. Ricci JE, Gottlieb RA, Green DR, Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis, *J. Cell Biol.* 160 (2003) 65–75. [PubMed: 12515825]
- [36]. Ricci JE, Munoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N, Scheffler IE, Ellisman MH, Green DR, Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain, *Cell* 117 (2004) 773–786. [PubMed: 15186778]
- [37]. Huai J, Vogtle FN, Jockel L, Li Y, Kiefer T, Ricci JE, Borner C, TNF α -induced lysosomal membrane permeability is downstream of MOMP and triggered by caspase-mediated NDUFS1 cleavage and ROS formation, *J. Cell Sci.* 126 (2013) 4015–4025. [PubMed: 23788428]
- [38]. Zhu Y, Li M, Wang X, Jin H, Liu S, Xu J, Chen Q, Caspase cleavage of cytochrome c1 disrupts mitochondrial function and enhances cytochrome c release, *Cell Research* 22 (2012) 127–141. [PubMed: 21577235]
- [39]. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ, Bax-deficient mice with lymphoid hyperplasia and male germ cell death, *Science* 270 (1995) 96–9. [PubMed: 7569956]
- [40]. Ali SS, Xiong C, Lucero J, Behrens MM, Dugan LL, Quick KL, Gender differences in free radical homeostasis during aging: short-lived female C57BL6 mice have increased oxidative stress, *Aging Cell* 5 (2006) 565–574. [PubMed: 17129217]
- [41]. Kokoszka JE, Coskun P, Esposito LA, Wallace DC, Increased mitochondrial oxidative stress in the SOD2^{-/-} mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis, *Proc. Natl. Acad. Sci. USA* 98 (2001) 2278–2283. [PubMed: 11226230]

- [42]. Armstrong D, Browne R, The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory, *Adv. Exp. Med. Biol* 366 (1994) 43–58. [PubMed: 7771281]
- [43]. Croteau DL, Stierum RH, Bohr VA, Mitochondrial DNA repair pathways, *Mutat. Res* 434 (1999) 137–148. [PubMed: 10486588]
- [44]. Gan W, Nie B, Shi F, Xu XM, Qian JC, Takagi Y, Hayakawa H, Sekiguchi M, Cai JP, Age-dependent increases in the oxidative damage of DNA, RNA, and their metabolites in normal and senescence-accelerated mice analyzed by LC-MS/MS: Urinary 8-oxoguanosine as a novel biomarker of aging, *Free Radic. Biol. Med.* 52 (2012) 1700–1707. [PubMed: 22348977]

- Oxidative stress increases with age in multiple organs.
- Mechanisms underlying this increase are little known.
- We report that Bax lies upstream of age-related increases in oxidative DNA damage in heart, liver, and kidney.
- Additionally, we find that caspase 3 lies upstream of age-related increases in oxidative DNA, lipid, and protein damage in these organs.
- These findings indicate that Bax and caspase 3, major components of the intrinsic apoptotic pathway, are central to the development of oxidative stress with age in these organs.

Fig. 1.

Bax deletion inhibited DNA damage in the aging mouse liver, heart, and kidney. **a** DNA damage in the liver was suppressed by *Bax* deletion. $n = 7$ -33 comets from three separate experiments. **b** DNA damage was also suppressed by *Bax* deletion in the aging heart. $n = 12$ -195 comets from three separate experiments. **c** *Bax* deletion suppressed DNA damage in the aging mouse kidney. $n = 165$ -185 comets from 3 separate experiments. Data is shown as fold change relative to the average in 5-month-old *Bax*^{+/+} animals based on percent DNA in tail. Within a genotype, different numbers in symbols indicate significantly different from

one or two of the other ages in that genotype while the same numbers indicate no difference. Different letters indicate significant difference between the three genotypes of that age while the same letters indicate no difference. $p < 0.001$ for indicated significant differences determined by Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's Multiple Comparison Test.

Fig. 2.

Genetic removal of *Casp3* suppressed oxidative stress in female 12-month-old mouse livers.

a Removal of *Casp3* suppressed 3-Nitrotyrosine (3-NT) levels in liver. Top: representative immunoblots showing higher 3-NT levels in the liver tissue of *Casp3*^{+/+} mice than in *Casp3*^{-/-} mice. Bottom: quantitative analysis of 3-NT band density demonstrated nitrated proteins were significantly decreased on depletion of *Casp3* ($p < 0.01$). Density was determined for entire lanes and was normalized to the immunoblot band density of β -actin ($n = 5-10$ mouse livers for each genotype). **b** Oxidation of liver DNA was greatly attenuated

in *Casp3*^{-/-} mice as compared to DNA oxidation in the livers of *Casp3*^{+/+} ($p < 0.01$). The results are expressed as the pg/ml of 8-OHdG for each group ($n = 7$ -10 livers per genotype). **c** TBARS assay was utilized to measure the levels of MDA, a lipid peroxidation product, in fresh liver tissues from mice with *Casp3*^{+/+} and *Casp3*^{-/-} genotypes. *Casp3* ablation significantly reduced lipid peroxide levels ($p = 0.01$; $n = 9$ -12 livers for each genotype). Asterisks indicate significantly different from wild type.

Fig. 3.

Casp3 ablation suppressed oxidative damage in 12-month-old female mouse hearts. **a** Accumulation of nitrated proteins in heart tissue was reduced on genetic deletion of *Casp3*. Top: representative immunoblots showing much greater levels of nitrated tyrosine products in the cardiac tissue of *Casp3*^{+/+} mice than in *Casp3*^{-/-} mice. Bottom: the differences in the 3-NT levels between the two genotypes were statistically significant ($p < 0.01$; $n = 5-10$ hearts for each genotype). Immunoblot densities of protein tyrosine nitration are normalized to the β -actin levels. **b** 8-OHdG levels were significantly suppressed in hearts from *Casp3*^{-/-}

mice ($p < 0.01$; $n = 7-10$ hearts for each genotype). **c** *Casp3* deletion significantly reduced lipid peroxidation in cardiac tissue as determined by the TBARS assay ($p < 0.01$; $n = 9-12$ hearts for each genotype). Asterisks indicate significantly different from wild type.

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Fig. 4.

Casp3 ablation suppressed oxidative damage in 12-month-old female mouse kidneys. **a** Top: immunoblots showing that 3-NT protein levels were much higher in kidneys from *Casp3*^{+/+} mice than in kidney from *Casp3*^{-/-} mice ($p < 0.05$; $n = 5-10$ kidneys per genotype). **b** 8-OHdG levels in DNA of the *Casp3*^{-/-} kidneys were significantly lower than in *Casp3*^{+/+} kidneys ($p < 0.01$; $n = 7-10$ kidneys per genotype). **c** Lipid peroxides were significantly

lower in the kidneys from *Casp3*^{-/-} mice than in those from *Casp3*^{+/+} mice ($p = 0.01$; $n = 9$ -12 kidneys for each genotype). Asterisks indicate significantly different from wild type.

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