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Burn-Induced Apoptosis in the Livers of Aged Mice is Associated with Caspase Cleavage of Bcl-xL

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Abstract

Background: Older adult burn victims have poorer outcomes than younger burn victims. The liver is critical for the recovery of patients with burns. Post-burn hepatic apoptosis in young individuals compromises liver integrity; however, this pathway has not yet been studied in older individuals. Because aged animals with burns suffer significant liver damage, we hypothesized that apoptosis is altered in these animals and may affect liver function. Understanding post-burn hepatic apoptosis and its effects on liver function in aged animals may help improve outcomes in older patients.

Materials and Methods: We compared the protein and gene expression levels in young and aged mice after a 15% total-body-surface-area burn. Liver and serum samples were collected at different time points after injury.

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Israel Muro, Juan-Pablo Idrovo, and Elizabeth J. Kovacs conceived and planned the experiments. Israel Muro, Andrea C. Qualman, and Juan-Pablo Idrovo carried out the experiments. Israel Muro, Elizabeth J. Kovacs, and Juan Pablo Idrovo contributed to the analysis and interpretation of the results. All authors provided critical comments and helped in the creation of the manuscript.

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The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript, and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Results: Caspase-9 expression in liver tissue was downregulated by 47% in young animals and upregulated by 62% in aged animals 9 h post-burn ($p<0.05$). The livers of aged mice showed a Bcl-xL transcription increase only after 6 h; however, the livers of young mice exhibited 4.3-fold, 14.4-fold, and 7.8-fold Bcl-xL transcription increases at 3, 6, and 9 h post-burn, respectively ($p<0.05$). The livers of young mice showed no changes in Caspase-9, Caspase-3, or Bcl-xL protein levels during the early post-burn period. In contrast, the livers of aged mice contained cleaved caspase-9, reduced full-length caspase-3, and an accumulation of ΔN-Bcl-x at 6 and 9 h post-burn ($p<0.05$). p21 expression decreased in aged mice; however, it was significantly increased in the liver tissue of young mice post-burn ($p<0.05$). Saa1 and Saa2 serum protein levels were 5.2- and 3.1-fold higher in young mice than in aged mice, respectively, at 6 and 9 h post-burn ($p<0.05$).

Conclusions: Livers of aged mice exhibited different apoptotic processes compared to those of young mice early after burn injury. Collectively, burn-induced liver apoptosis in aged mice compromises hepatic serum protein production.

Keywords

liver; burn; trauma; aging; apoptosis; Bcl-xL

1. Introduction

Individuals >65 years of age comprise nearly 10% of the world's population, and this number is expected to double by 2050 (1). In the United States, approximately 15% of the population is >65 years of age, and in 40 years, it is expected be closer to 25% (2). Burn injuries are a devastating form of trauma; nationwide, older victims of burns account for 20% of patients admitted to burn centers (3, 4). Moreover, older patients with burns exhibit an overall mortality rate of >40% compared with younger patients with similar injuries (5). This age-related disparity in outcomes and rising population of older adults underscore the need to develop age-targeted treatments.

The liver plays a critical role in the survival and recovery of burn injury victims through the regulation of several metabolic, inflammatory, immunological, and acute phase processes (6). The production of acute-phase proteins (APPs) is a pivotal liver function in burn injuries (6, 7). APPs such as serum amyloid A1 (Saa1) and serum amyloid A2 (Saa2) are critical for regulating metabolism, immunity, and inflammation (8, 9). Severe burns in young adults affect liver integrity and jeopardize patient outcomes (10, 11). Burn injury exerts an intense impact on liver function, and studies in young animals have shown that this stress leads to hepatic apoptosis and decreased liver function (12–15). Apoptosis is a genetically regulated form of cell death that eliminates damaged and unwanted cells. Cysteinyl aspartic proteases (caspases) are executioners of apoptosis and are produced as full-length inactive proteins that require proteolytic processing for activation (16–18). Caspase processing is controlled by two separate pathways, namely the extrinsic and intrinsic cell death pathways (19, 20). The extrinsic pathway induces caspase-8 processing and is activated by ligand binding to plasma transmembrane receptors, collectively known as death receptors (19). Mitochondrial outer membrane permeabilization (MOMP) activates the intrinsic pathway and promotes caspase-9 processing by releasing proapoptotic factors (20). Caspase-8 and −9 are initiator caspases; once active, they cleave and activate caspase-3. As an effector caspase, caspase-3

cleaves essential cellular components and kills cells (18). The B-cell lymphoma-2 (Bcl-2) family consists of pro- and anti-apoptotic proteins that influence apoptosis by regulating MOMP (21, 22). BAX, BAK, and BOK are proapoptotic Bcl-2 proteins that induce apoptosis by forming pores along the MOM and triggering MOMP. Bcl-2, Bcl-xL, and Mcl-1 are anti-apoptotic Bcl-2 family members that directly bind to pro-apoptotic proteins to prevent pore formation. Interestingly, Bcl-xL contains a caspase cleavage site near the N-terminus, and when cleaved, generates ΔN-Bcl-xL. In contrast to Bcl-xL, ΔN-Bcl-xL is pro-apoptotic and can form pores in the outer mitochondrial membrane to promote MOMP (18–22).

Although hepatic apoptosis has been widely studied in young burn victims, a significant knowledge gap exists regarding post-burn hepatic apoptosis in the older adult population. We previously demonstrated that the livers of older mice show considerable hepatic stress following burn damage compared to young animals (23). Because burn-induced liver stress leads to hepatic apoptosis in young mice, we hypothesized that elevated liver stress in aged burned mice relative to that in young burned animals would lead to higher rates of hepatic apoptosis (12–15). Furthermore, increased levels of hepatic apoptosis may cause the livers of aged mice with burns to function less efficiently than those of young mice with burns.

Thus, we examined post-burn hepatic apoptosis in young and aged mice by comparing changes in the liver tissue at different time points after burn injury. Liver function was compared by measuring the APP protein production (Saa) among the study groups, as APPs are the primary type of protein produced by the liver early after burn injury. We used a well-established murine burn model (24–26) to conduct these studies.

2. Materials and methods

2.1. Mice

Young (8–10 weeks old, equivalent to 20–25 human years) and aged (20–21 months old, equivalent to 65–70 human years) female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and the National Institute of Aging (NIA) Colony (Charles River Laboratories, Wilmington, MA, USA), respectively. The mice were acclimatized at the University of Colorado Anschutz Medical Campus Vivarium for at least 2 weeks before performing the experiments. To minimize circadian rhythm variability, all burn experiments were performed between 9 and 11 am. All procedures were approved by the University of Colorado Institutional Animal Care and Use (IACUC protocol no. 00001163).

2.2. Burn injury model

The mice were randomly assigned to four experimental groups (young sham, young burn, aged sham, and aged burn), with n=10 per group per time point. To create a 15% full-thickness total body surface area (TBSA) burn, mice were anesthetized with 55.5 mg/kg of ketamine and 2.6 mg/kg of xylazine (Webster Veterinary, Sterling, MA). The dorsum of each mouse was shaved based on the mass and placed in templates to uncover the proper amount of skin. Full-thickness scald burns were achieved by treating the mice with boiling water at

95 °C for 10 s (27). The mice in the sham group were exposed to water at room temperature for 10 s. After treatment, mice received separate intraperitoneal injections of 0.13 mg/kg of buprenorphine for pain management and 1 mL of normal saline for resuscitation. Blood was collected immediately after the mice were sacrificed at 3, 6, and 9 h post-burn. Blood was centrifuged at 10,000 × g for 5 min at 4°C to collect the serum. The livers were harvested at 3, 6, and 9 h post-burn and snap-frozen in liquid nitrogen. Serum and liver samples were stored at −80 °C prior to use. The livers were maintained in tubes on dry ice after the pieces were removed for homogenization. The homogenized tissues were stored in tubes on ice prior to analysis.

2.3. Serum amyloid enzyme-linked immunosorbent assay (ELISA)

Serum Amyloid A1 and A2 (Saa1 and Saa2) protein levels were measured in mouse serum using a Mouse Serum Amyloid A DuoSet ELISA kit (R&D Systems, no. DY2948–05). As Saa1 and Saa2 share 91% homology in their amino acid sequences, the ELISA antibodies simultaneously detected both proteins (28). The ELISA was performed according to the manufacturer's instructions (29).

2.4. Quantitative polymerase chain reaction (qPCR)

RNA was extracted from liver tissue using the RNeasy Mini Kit (no. 74106, Qiagen). The iScript cDNA synthesis kit (no. 1708891, BioRad) was used to generate cDNA, as previously described (23). TaqMan probes and TaqMan Universal PCR Master Mix (no. 4304437) were purchased from Fisher Scientific and used for the qPCR analysis of Saa1 (no. Mm00656927), Saa2 (no. Mm04208126), haptoglobin (no. Mm00516884), and fibrinogen (no. Mm00805336) levels. For Caspase-9, Caspase-3, Bcl-2, Bak, Bax, and Bcl-xL, qPCR was performed using Universal SYBR Green Supermix (Bio-Rad, no. 1725124). Standard desalted primers were purchased from Sigma-Aldrich. Caspase-9 Forward Primer: GCTGTGTCAAGTTTGCCTACCC, Caspase-9 Reverse Primer: CCAGAATGCCATCCAAGGTCTC; Caspase-3 Forward Primer: GGAGTCTGACTGGAAAGCCGAA, Caspase-3 Reverse Primer: CTTCTGGCAAGCCATCT-CCTCA; Bcl-2 Forward Primer: CCTGTGGATGACTGAGTACCTG, Bcl-2 Reverse Primer: AGCCAGGAGAAATCAAACAGAGG; Bak Forward Primer: GGAATGCCTACGAACTCTT-CACC, Bak Reverse Primer: CAAACCACGCTGGTAGACGTAC; Bax Forward Primer: AGG-ATGCGTCCACCAAGAAGCT, Bax Reverse Primer: TCCGTGTCCACGTCAGCAATCA; and Bcl-xL Forward Primer: GCCACCTATCTGAATGACCACC, Bcl-xL Reverse Primer: AGGA-ACCAGCGGTTGAAGCGC. GAPDH was used as an endogenous control (no. 4352339E; Fisher Scientific).

2.5. Western blotting

Mouse livers were homogenized and sonicated in lysis buffer containing 20 mM Tris (pH 7.4), 150 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail, as previously described (30). Protein concentrations were determined using the Protein Assay Dye Reagent (no. 5000006, BioRad), and 20 μg of protein was run on

polyacrylamide gels (no. 4568085, BioRad) for analysis. Proteins were transferred using a Nitrocellulose Transfer Kit (no. 1704270; Bio-Rad) and a Trans-Blot Turbo Transfer System (#1704150; Bio-Rad). The membranes were blocked with blotting buffer (no. 12010020, Bio-Rad). Membranes were incubated with primary antibodies overnight at 4°C in 1X TBS (no. 1706435, BioRad) with 0.1% Tween-20 (Sigma, P1379) and 2% bovine serum albumin (BSA; no. B2518, Sigma). The primary antibodies used were GAPDH-HRP (no. 51332, Cell Signaling), Caspase-9 (no. 9504, Cell Signaling), Caspase-3 (no. 9662, Cell Signaling), Bcl-xL (no. 10783–1-AP, ProteinTech), and p21 (no. 64016, Cell Signaling). Horseradish peroxidase (HRP)-linked anti-rabbit IgG was used as the secondary antibody (Cell Signaling #7074). ECL Western Blotting Substrate (no. 32106, Fisher Scientific) was used for chemiluminescence, and blots were visualized on a ChemiDoc Imaging System (no. 12003153, Bio-Rad). Densitometry was performed with GAPDH normalization to each of the target proteins using Image Lab 6.1.

2.6. Statistical analysis

Power analyses were performed using Colorado Biostatistics Consortium software. The consortium is a unit of the Department of Biostatistics and Informatics, Colorado School of Public Health, University of Colorado, Denver, USA. Data are presented as boxplots with n = 10 per group at each time point. The sample size estimate was centered on data previously collected based on previous studies employing this model and the drastic difference in outcomes between young and aged mice in our burn injury model (23, 31, 32). Data were analyzed using GraphPad Prism 8.4.1 software, with one-way analysis of variance (ANOVA), followed by Tukey's test for post hoc statistical comparisons between multiple groups, where ($p < 0.05$) was considered significant. Continuous data were evaluated for distribution normality in the Praph Pad Prism program using column statistics, the Shapiro-Wilk Test and Q-Q pots. The Brown–Forsythe test was used to test the homogeneity of variance.

The ARRIVE guidelines were used to ensure proper reporting of methods, results, and discussions.

3. Results

3.1. Relative to young mice, the livers of aged mice display a proapoptotic expression profile after burn injury

Induction of the extrinsic pathway activates caspase-8, whereas caspase-9 is activated through the intrinsic pathway (19, 20). The overexpression of caspase-8 and caspase-9 triggers apoptosis (17, 33). To determine whether burn injury alters the transcription of *Caspase-8* or *Caspase-9*, we performed qPCR analysis on livers from mice 3, 6, and 9 h post-burn. These time points were selected to associate alterations in gene expression with an early response to burn injury (34). Up to 9 h post-burn, *Caspase-8* expression remained at near basal levels in the livers of both young and aged mice (Figure 1A). Three and six hours after burn injury, *Caspase-9* also remained unchanged in the livers of young and aged animals (Figure 1B). At 9 h post-burn, livers from young mice displayed a 47% decrease ($p<0.0001$) in *Caspase-9* expression compared to that in sham-treated young mice (Figure

1B). The livers of aged animals responded in an opposite manner to those of young mice and at 9 h post-burn demonstrated a 62% increase ($p<0.05$) in *Caspase-9* expression relative to that in sham-treated aged mice (Figure 1B).

As changes in *Caspase-9* expression suggest the involvement of the intrinsic apoptotic pathway, we examined the transcription of Bcl-2 proteins, including the anti-apoptotic proteins *Bcl-2* and *Bcl-extra-large* (*Bcl-xL*), as well as the pro-apoptotic *Bcl-2 homologous antagonist killer* (*Bak*) and *Bcl-2-associated X protein* (*Bax*). Throughout the burn time course, only minor changes in *Bcl-2*, *Bak*, and *Bax* transcription were observed in the livers of young and aged mice (Figure 2A, C, and D). Conversely, the burn injury-induced upregulation of *Bcl-xL* in the livers of young mice compared to young sham animals was elevated by 4.3-fold ($p<0.0001$) at 3 h, 14.4-fold ($p<0.001$) at 6 h, and 7.8-fold ($p<0.01$) at 9 h (Figure 2B). *Bcl-xL* expression in the livers of aged mice remained unchanged at 3 h post-burn, was 9.6-fold ($p<0.05$) higher than that in sham-treated aged animals at 6 and by 9 h returned to basal levels (Figure 2B).

3.2. Compared to young mice, aged mice exhibit aberrant liver apoptosis earlier after burn injury

To confirm apoptosis, liver lysates were evaluated using western blot analysis using antibodies directed against the full-length and processed forms of caspase-9 and caspase-3. As the livers of young mice showed a significant increase in Bcl-xL expression after burn injury, the liver lysates were probed for changes in Bcl-xL protein levels. Three hours after the burn injury, the livers of young and aged mice contained only full-length caspase-9 and caspase-3 and demonstrated no changes in Bcl-xL protein (Figure 3A and B). At 6 and 9 h post-burn, livers from young animals showed no evidence of apoptosis, as there was no detectable cleavage of caspase-9 or caspase-3, and the Bcl-xL protein was unchanged (Figure 4). Apoptosis was confirmed in the livers of aged mice by the significant production of a caspase-9 cleavage product compared to all other groups ($p<0.05$) 6 and 9 h after burn injury (Figure 4A and B). Caspase-3 analysis demonstrated that livers containing active caspase-9 ($p<0.05$) had lower levels of full-length caspase-3. Although we were unable to detect cleaved caspase-3, this finding was not unexpected, as cleaved caspase-3 was rapidly degraded (Figure 4C and D) (35). Furthermore, transcriptional analysis of caspase-3 in the liver showed that burn injury had minimal effect on caspase-3 expression in both young and aged animals (Supplementary Figure 1). Surprisingly, analysis of Bcl-xL revealed accumulation of the Bcl-xL caspase cleavage product, N-Bcl-xL, in the livers of aged mice that displayed active caspases ($p<0.05$) compared to all other groups (Figure 4E and F). Notably, although full-length Bcl-xL is anti-apoptotic, N-Bcl-xL is proapoptotic (36).

3.3. The cell cycle inhibitor, p21, is a putative regulator of caspase-mediated cleavage of Bcl-xL

Caspase cleavage of Bcl-xL is governed by cyclin-dependent kinase 2 (CDK2), a serine/threonine protein kinase that phosphorylates Bcl-xL to promote its cleavage into N-Bcl-xL (37, 38). CDK2 supports cell division and is potently inhibited by cell cycle inhibitor p21 (39). Because p21 is also known to regulate Bcl-xL activity, we examined the livers of young and aged mice to evaluate the potential role of p21 in controlling Bcl-xL cleavage

(40, 41). Analysis of the p21 protein in sham-treated young and aged mice showed differential expression, with p21 expression being higher in the livers of aged animals (Figure 5A and B). At 6 and 9 h post-burn young mice upregulate p21 protein was upregulated 9-fold and 19-fold ($p<0.05$), respectively, in young mice compared to that in sham-treated young animals (Figure 5A and B). Contrary to the livers of young mice, the livers of aged mice downregulated p21 protein and by 9 h post-burn p21 protein was 75% below basal levels ($p<0.05$) and 93% lower than that of livers from young burned mice ($p<0.05$) (Figure 5A and B).

3.4. Livers from aged mice exhibit a reduced response to burn injury compared to young mice

In response to burn injuries, the liver produces large quantities of APPs to restore homeostasis following their release into the bloodstream (9). Saa1 and Saa2 are the two major APPs measured in the mouse serum to examine liver function (42). At 3 h post-burn, the serum of young and aged mice had similar levels of Saa1 and Saa2 as those of young and aged sham animals, respectively. However, 6 h after burn injury, young animals showed a 4-fold increase ($p<0.0001$) in serum Saa1 and Saa2 protein levels compared to young sham animals. Unlike young mice, aged mice failed to show elevated Saa1 or Saa2 serum protein at 6 h post-burn. It was not until 9 h after the burn injury that the serum of aged mice contained levels of Saa1 and Saa2 proteins above those observed in aged sham animals. At this time point, Saa1 and Saa2 serum protein levels were modest but significant 2.7-fold than the sham levels ($p<0.05$). Nine hours after burn injury, serum from young mice showed an 8.4-fold rise ($p<0.0001$) in Saa1 and Saa2 protein levels. Lastly, it should be noted that at 9 h post-burn, Saa1 and Saa2 protein levels in the serum of young mice were 3.1-fold higher ($p<0.001$) than those in the serum of aged mice (Figure 6).

4. Discussion

Burn injury is a highly demanding condition that induces multi-organ stress, and excessive tissue demand can induce apoptosis, causing further complications. Aberrant apoptosis is problematic and may lead to detrimental changes in tissue and organ integrity (43). Compared with young animals, the livers of aged mice exhibit significant deterioration after burn injury (23). As apoptosis plays an important role in post-burn liver pathophysiology in young adult rodents, we hypothesized that the livers of aged animals may be further compromised after burn injury because of disproportionate apoptosis (12–15). Therefore, we performed this study to analyze and compare liver apoptosis after burn injury in young and aged mice.

Our examination of the early stages after burn injury revealed that up to 9 h post-burn, the livers of young mice showed no evidence of apoptosis (Figures 3 and 4). This finding is consistent with a previous report that evaluated the early response to burn injury in young mice and failed to detect liver apoptosis 3 h post-burn (44). Interestingly, the livers of young mice have been shown to contain modest levels of apoptosis at 24 h post-burn and at later time points (12–15). Our investigations at earlier time points after burn injury indicated that the livers of aged mice display burn-induced apoptosis earlier than those of

younger mice (Figure 4). The rapid appearance of hepatic apoptosis suggests that, relative to young mice, the livers of aged mice prematurely succumb to the stress of burn injury. A comparison of liver apoptosis at later time points post-burn is necessary to thoroughly define the age-specific progression of hepatic stress and liver apoptosis throughout the recovery process.

Characterization of burn-induced liver apoptosis in aged mice revealed the accumulation of the Bcl-xL caspase cleavage product, N-Bcl-xL (Figures 4E and F). An *in vitro* study using purified proteins originally identified Bcl-xL as a caspase substrate and determined that it is cleaved at aspartate 61 to generate N-Bcl-xL (36). Furthermore, the same study performed overexpression experiments of N-Bcl-xL using hamster kidney cells and demonstrated that, in contrast to Bcl-xL, N-Bcl-xL is pro-apoptotic. More recently, investigations using different *in vivo* and *in vitro* models of cerebral ischemia have shown that N-Bcl-xL production is vital for triggering neuronal apoptosis (36, 45–47). Research on the regulation of Bcl-xL has identified CDK2 as a kinase that phosphorylates Bcl-xL and promotes its production (37). Since p21 is a potent inhibitor of CDK2, our finding that after burn injury, p21 protein is elevated as high as 19-fold in the livers of young mice but is reduced by 75% in the livers of aged mice suggests an age-related model for controlling the cleavage of Bcl-xL (Figure 5). Higher levels of p21 protein in the livers of young mice are expected to block CDK2 phosphorylation of Bcl-xL, thereby preventing the production of N-Bcl-xL. In contrast, low levels of p21 in the livers of aged mice would permit CDK2 phosphorylation of Bcl-xL and augmented the generation of N-Bcl-xL. Although future studies are needed to identify how p21 is differentially regulated in young and aged mice, our data suggest that the production of N-Bcl-xL in the livers of aged mice is a feed-forward mechanism that lowers the threshold for apoptotic activation.

Having found that the livers of aged mice exhibited a characteristic apoptotic pattern post-burn, we explored its effects on the liver function. Saa1 and Saa2 are the major APPs produced by the liver early after a burn injury. Our finding that aged mice failed to fully upregulate Saa1 and Saa2 serum proteins suggests that aberrant liver apoptosis hampers Saa1 and Saa2 protein production (Figure 6). As Saa1 and Saa2 are critical for modulating cholesterol efflux, attracting and supporting neutrophils, activating macrophages, and promoting pathogen clearance, inadequate Saa1 and Saa2 production is expected to negatively affect recovery process (48). Future investigations evaluating the effect of inhibiting liver apoptosis are necessary to assess the role of burn-induced apoptosis on liver function and APP production. Since N-Bcl-xL is only produced under certain apoptotic conditions, targeting N-Bcl-xL would be an excellent strategy for blocking burn-induced liver apoptosis, as it would minimize the pleiotropic effects on general apoptosis. Collectively, our research suggests an age-specific mechanism for controlling liver apoptosis after burn injury and identifies caspase-mediated cleavage of Bcl-xL as a network node for developing age-targeted therapies in patients with burns.

Murine models do not always replicate clinical observations. However, our group found multiple parallels between clinical observations in patients with burns and the murine models examined herein in terms of circulating inflammatory mediators and multi-organ damage resulting from a moderate-sized cutaneous scald injury (25, 49–52). We

acknowledge the limitations of the murine model, as the animals were genetically identical and resided in a controlled environment. The anatomy and physiology of rodent skin differs from those of humans, and inflammatory mediators in the affected skin may vary in quality and quantity from human skin. Burn victims are treated in a burn intensive care unit (ICU), where continuous intravenous fluids, antibiotics, nutrition, and monitoring are provided; however, the lack of a rodent ICU creates a drastic difference in the treatment environment, adding additional variables that can affect the results of experiments. Environmental variables come from different mouse vendors and can affect the results of the experiments. The difference in weight between the young and aged mice in this study was approximately 3–5 grams which may have affected our results. The half-life of caspases can be affected by factors such as protein-protein interactions, post-translational modifications, and cellular stress conditions impacting the results. This study was performed using female mice because publications have demonstrated higher mortality rates in women who sustain burn injuries than in men. This is an important limitation, as the results obtained may only be pertinent to a part of the population (53).

In conclusion, these findings suggest that the livers of aged mice undergo a unique apoptotic process compared to the livers of younger animals early after burn injury. Furthermore, burn-induced liver apoptosis in aged mice may influence hepatic serum protein production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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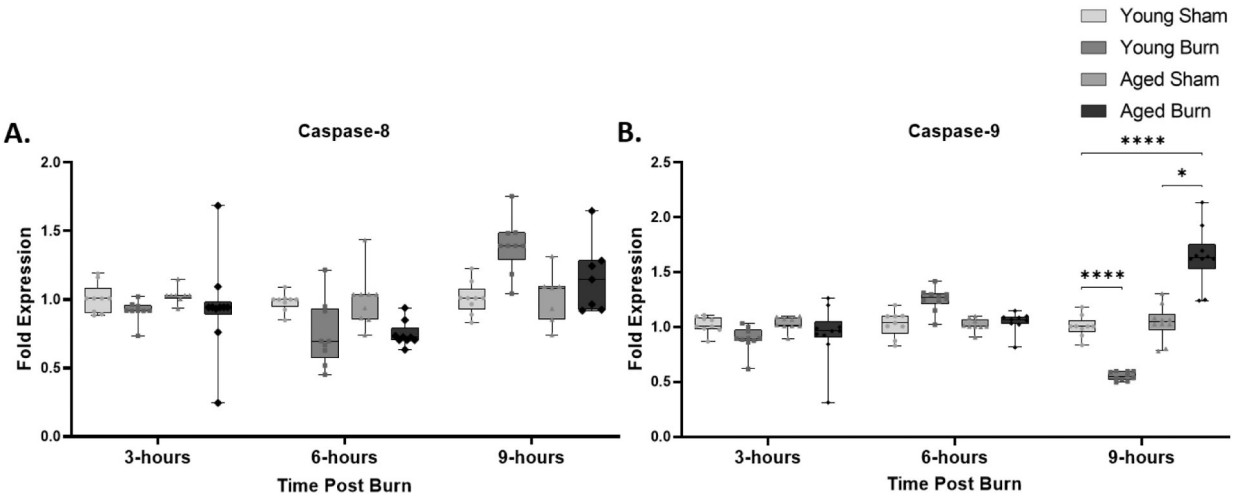

Figure 1. Opposing effects of Caspase-9 transcription in the livers of young and aged mice after burn injury.

Whole livers were analyzed using qPCR. (A) Caspase-8 (B) Caspase-9 Results were determined using the ΔΔCt algorithm with GAPDH as an internal control. Data are presented as boxplots. n = 10 per group; *p<0.05, ****p<0.0001

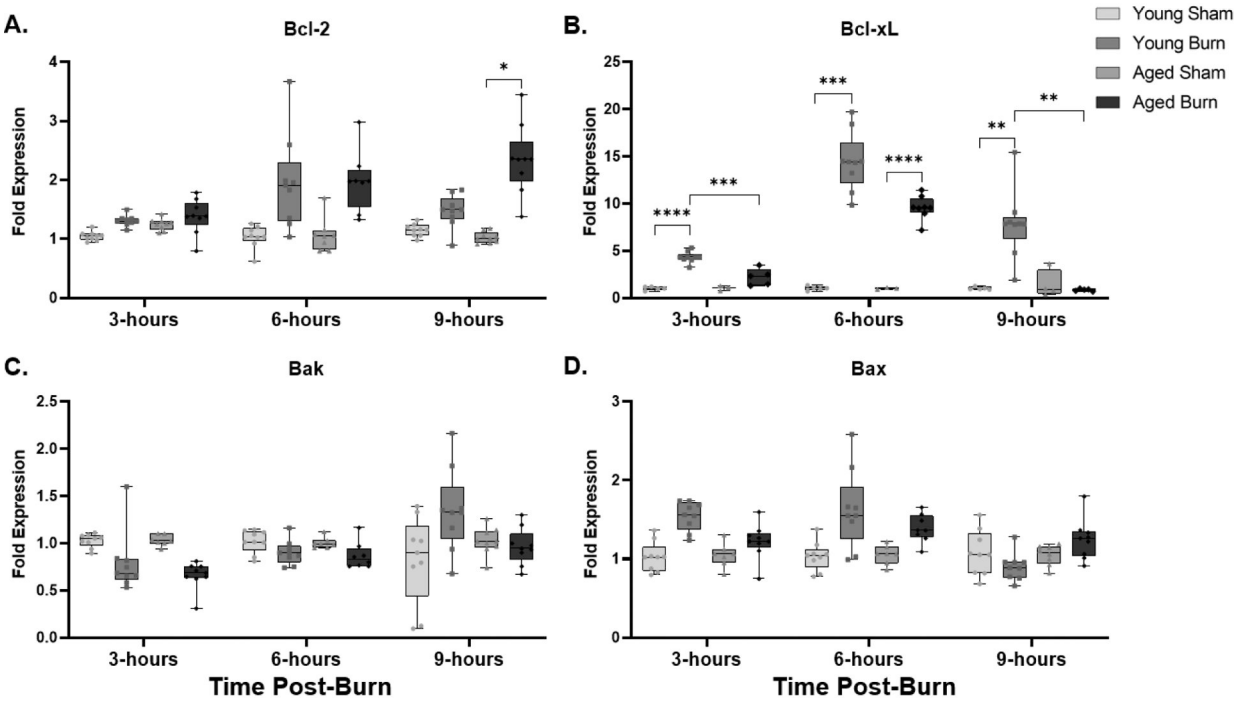

Figure 2. Expression of Bcl-2 family members in livers from mice post-burn.

Whole livers were analyzed using qPCR. (A) Bcl-2 (B) Bcl-xL (C) Bak (D) Bax Results were determined using the ΔΔCt algorithm with GAPDH as an internal control. Data are presented as boxplots. n = 10 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

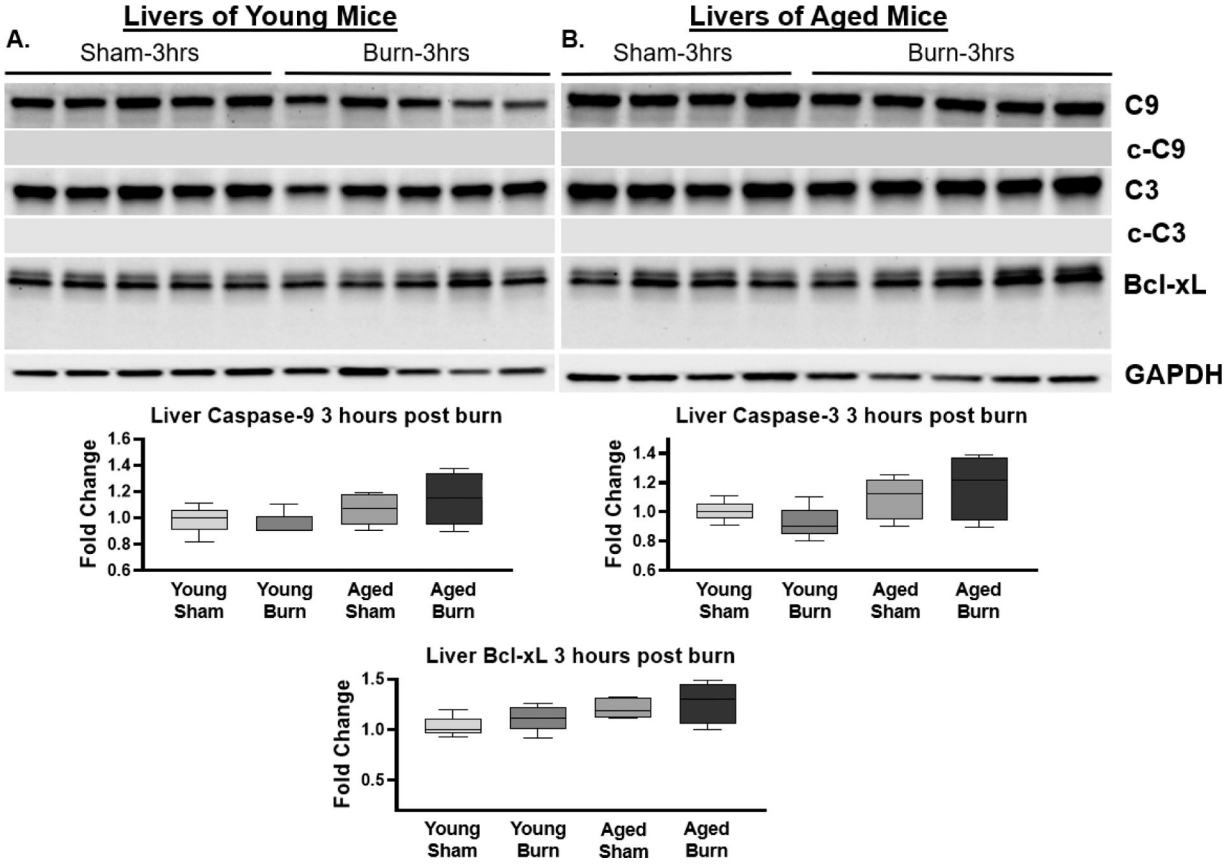

Figure 3. Apoptosis is not detected 3 h post-burn in livers from young or aged mice.

Western blot analysis was performed on liver lysates using antibodies specific to caspase-9 (C9), caspase-3 (C3), or Bcl-xL. Cleavage Caspase 9= c-C9, Cleavage Caspase 3= c-C3. GAPDH was used as a loading control. Densitometry was performed using GAPDH normalization to each one of the target proteins. Data are presented as boxplots. Representative images are shown, n= 10

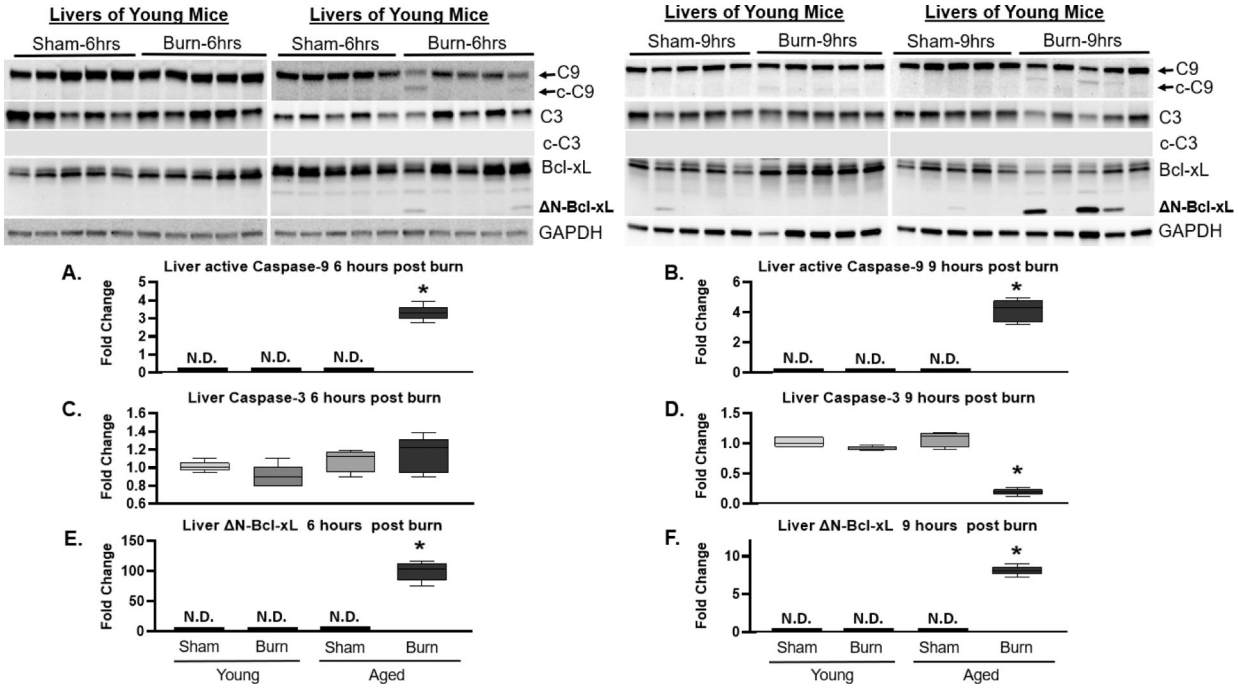

Figure 4. Apoptosis is detected in the livers of aged mice 6 and 9 h post-burn.

Liver lysates were probed at the indicated time points with antibodies specific to caspase-9 (C9), caspase-3 (C3), or Bcl-xL. GAPDH was used as a loading control. (A) Active Caspase-9 6 h post-burn, (B) Active Caspase-9 9 h post-burn, (C) Caspase-3 6 h post-burn, (D) Caspase-3 9 h post-burn, (E) ΔN-Bcl-xL 6 h post-burn, and (F) ΔN-Bcl-xL 9 h post-burn. Protein not detected (N.D.). Cleavage Caspase 9= c-C9, Cleavage Caspase 3= c-C3. Densitometry was performed using GAPDH normalization to each one of the target proteins. Data are presented as boxplots. Representative images are shown, n= 10 per group *p<0.05

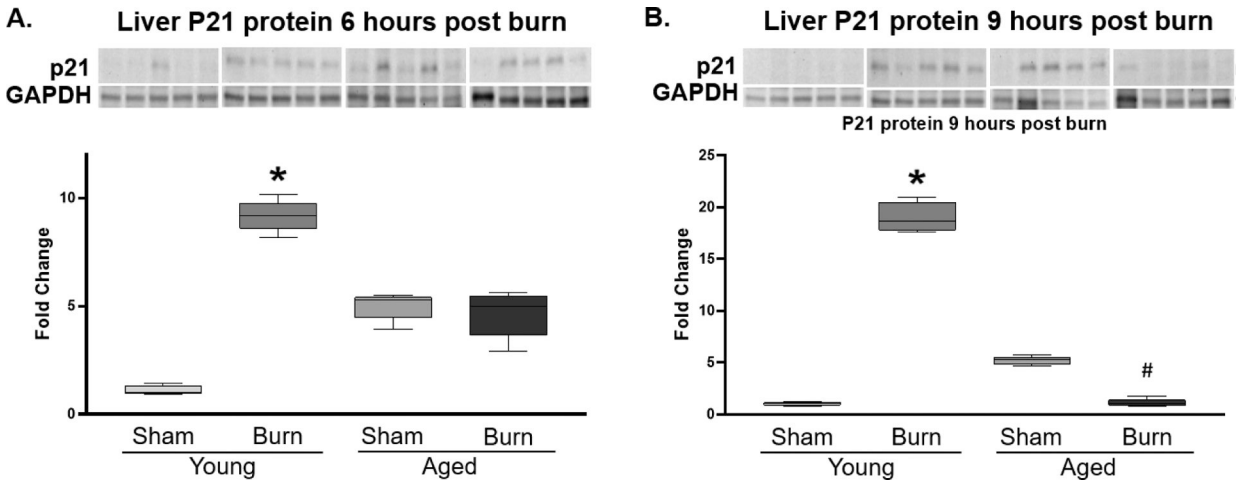

Figure 5. The livers of young and aged mice exhibit differential expression of p21 protein.

Liver lysates were analyzed at 6 and 9 h post-burn for p21 using western blotting. GAPDH was used as a loading control. Densitometry was performed using GAPDH normalization to each one of the target proteins. Data are presented as boxplots. Representative images are shown, n= 10 per group *p<0.05 compared to all other groups. p<0.05 compared to aged sham and young burn groups.

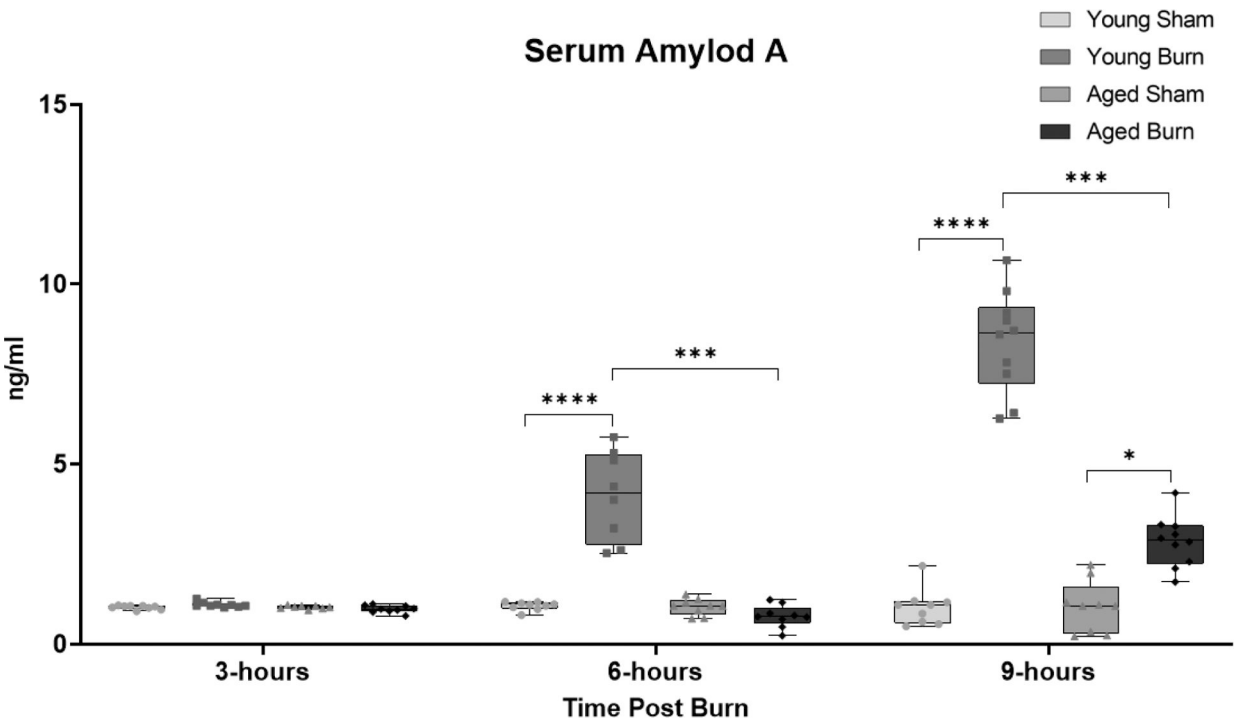

Figure 6. Induction of Serum SAA in the Serum is reduced in aged mice after burn injury.

Serum SAA protein was measured using ELISA. SAA protein quantities are presented as ng/mL of Serum. Data are presented as boxplots. SAA fold change compared to the age-matched sham group. n = 10 per group; *p<0.05, ***p<0.001, ****p<0.0001