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Simvastatin protects hepatocytes from apoptosis by suppressing the TNF- α /caspase-3 signaling pathway in mice with burn injury

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INTRODUCTION

There is increasing evidence that the liver is one of the main targets for organ dysfunction after thermal injury. Most of patients suffering from severe burn trauma also have hepatic dysfunction [1, 2]. However, it is not clear whether thermal injury induces programmed cell death in the remote liver, or if an imbalance between hepatic cellular proliferation and apoptosis leads to homeostatic dysfunction of the liver [3, 4].

Simvastatin, a 3-hydroxy 3-methyl-glutaryl coenzyme-A (HMG-CoA) reductase inhibitor, has clinically been shown to reduce hepatic complications, including hepatic dysfunction and failure in severely burned patients [5]. These effects of statins have been shown to be related both directly and indirectly to blocking HMG-CoA reductase activity [6, 7]. Simvastatin interferes with the synthesis of farnesylpyrophosphate. Farnesylpyrophosphate is needed for many biosynthetic pathways, including the synthesis of ubiquinone, a component of the mitochondrial respiratory chain, and post-translational lipidation of proteins. These pathways are all involved in cellular apoptosis [8]. However, the underlying mechanism(s) remain obscure.

The primary use of statins is to treat hypercholesterolemia [9]; however, accumulating data suggest that statins also exert anti-inflammatory effects by decreasing the formation of pro-inflammatory cytokines, chemokines, and reactive oxygen species [10, 11]. Burn injury can induce significant inflammatory reactions in the liver, which may trigger or promote hepatic cellular apoptosis. However, it is not known whether statins suppress susceptibility to burn-induced hepatic apoptosis via their anti-inflammatory properties. Clinically, statins have been shown to decrease the morbidity and mortality rate in burn patients which is attributable to reduction of liver complications [12]. Therefore we hypothesize that the protective role of statins on liver is related to a reduction of apoptosis and it is necessary to evaluate the effects of statin on hepatocyte apoptosis.

Tumor necrosis factor- α (TNF- α), a key mediator of the effects of burn injury, has been shown to promote leukocyte recruitment and to induce hepatocyte apoptosis in some disease

Competing interests

None.

conditions [13, 14]. TNF- α initiates cellular apoptosis as a potent extracellular stimulator. Downstream of the apoptotic TNF- α signaling pathway, caspase-3 plays a crucial role in the guidance of cells to undergo apoptosis [15]. Cleavage of procaspase-3 leads to active caspase-3 expression. Furthermore, Slotta and colleagues have found that simvastatin can reduce TNF- α expression and apoptosis in endotoxin-induced liver injury [16, 17]. It is not clear if burn injury promotes TNF- α expression in the liver or if simvastatin affects hepatocellular apoptosis via the TNF- α /caspase-3 pathway.

In the present study, we hypothesized that treatment with simvastatin reduces burn-induced apoptosis and may exert this anti-apoptotic activity by reducing pro-inflammatory cytokines, specifically, TNF- α and caspase-3. To test this hypothesis, we used an experimental model in which mice were exposed to thermal injury and then treated with simvastatin.

METHODS AND MATERIALS

Materials

TNF- α inhibitor: Pentoxifyline, Ketamine and Xylazine were from Sigma-Alorich (Louis, MO, U.S.A). Caspase-3 inhibitor: Ac-DEVD-CHO (C20H30N4O11) was from Alexis Biochemicals (San Diego, CA, U.S.A). Anti-TNF antibody, anti-active caspase-3 antibody, and anti-GAPDH antibody were from Cell Signaling (Danvers, MA, U.S.A). Cell Death Detection kit was obtained from Roche Molecular Biochemicals (Mannheim, Germany). DC protein assay kit was obtained from Bio-Rad (Hercules, CA). Polyvinylidene difluoride membranes were obtained from Amersham Biosciences (Buckinghamshire, UK). Collagenase was obtained from Sigma Aldrich (St. Louis Mo, U.S.A). DMEM, fetal calf serum and Penicillin/streptomycin were obtained from GIBCO (NY).

Animals

Wide-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were divided into three groups: Sham burn, burn with saline treatment and burn injury with Simvastatin treatment. The extent of hepatic apoptosis was evaluated in these animals. For further evaluation of the protective effect of Simvastatin in relation to inflammatory status. Animals were treated with TNF- α inhibitor (Pentoxifyline) and Caspase 3 inhibitor (Ac-DEVD-CHO). The effects of Simvastatin were also measured in a hepatic cell culture system. Finally, studies were also conducted on TNF- α $-/-$ and Caspase 3 $-/-$ (C57BL/6 genetic background, Jackson Laboratory) animals, to further explore the effects of Simvastatin and inflammation mediators on apoptosis. The study was approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Harvard University, and in compliance with the "Guide for the Care and Use of Laboratory Animals" (Publication No. NIH 78-23, 1996).

Burn injury model

Male mice weighing 20–25g were used in the present study. As previously described in reports from Shriners Burns Hospital laboratory [18], all animals received general anesthesia (Ketamine 40mg/kg body weight and Xylazine 5 mg/kg body weight, IP) prior to burn injury. A full-thickness thermal injury of 30% total body surface area (TBSA) was produced by shaving the dorsal surface of the animals with animal hair clippers. The animals were

then placed in molds exposing 30% of the dorsum followed by exposure of the open area to a 90°C water bath for 9 seconds. The mice were immediately resuscitated with 1.5 ml saline by intraperitoneal injection. Sham control mice were treated with a similarly; however the water bath was set to room temperature. After the procedure, the mice were caged individually.

Immediately following burn injury, the simvastatin-treated mice were injected with 100 µg/kg of simvastatin intraperitoneally and a second dose was administered 12 hours later. Intraperitoneally, the inhibitor-treated mice were administered 50 µg/kg of TNF-α inhibitor (Pentoxifyline) or 50 µg/kg of caspase-3 inhibitor (Ac-DEVD-CHO) immediately after injury and a second dose was administered 12 hours later on the day of the procedure. In the control group, sham treated mice received the same volumes of saline.

Histopathological Examination

The mouse liver specimens were fixed in 10% formaldehyde and embedded in paraffin. 5 µm tissue sections were stained with hematoxylin and eosin and then examined with light microscopy by experienced pathologists, unaware of the treatment conditions, to detect the histopathological changes (5 mice in each group).

Evaluation of Hepatocyte Apoptosis

4 µm liver sections were deparaffinized in xylene and dehydrated in graded ethanol. Apoptotic cells were identified using Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) kit. Ten random fields from 3 slides per group were examined and the TUNEL-positive brown nuclei within the hepatocytes were scored as previously described [19]. The data were expressed as the percentage of TUNEL-positive cells/100 hepatocytes. Digitized images were analyzed by two blinded investigators.

In the time course study of hepatocyte apoptosis, the liver specimens were collected at 6, 12, 24, 72, and 144 hours (5 mice in each time point) after thermal injury, and TUNEL staining was used for evaluation of apoptosis.

Western Blot Analysis and time course study on TNF-α and caspase-3 expression in liver

Total hepatic protein was extracted and concentration was measured by the Bradford method. Equal amounts of protein from each sample were loaded onto 10% SDS-PAGE and transferred onto equilibrated polyvinylidene difluoride membranes by electroblotting. The membranes were incubated overnight at 4°C with primary antibodies. After incubation with the secondary antibody, proteins were detected by enhanced chemiluminescence kit (ECL, Amersham Corporation). The level of expression of each protein was normalized to the expression level of GAPDH (a house keeping gene) in the same sample. Bands were quantified by scanning densitometry using the digital Kodak Gel Logic 200 system (Carestream Molecular Imaging, USA).

In the time course study of gene expression, liver specimens were collected at 6, 12, 24, and 72 hours after the thermal injury. Sham group: n=4, other groups: n=6 (the experiment was

repeated once). Liver proteins were isolated and western blot analysis of TNF- α and caspase-3 expression was performed.

Primary Culture of Hepatocytes and Treatment

Mouse hepatocytes were isolated by a collagenase perfusion technique according to previous methods with minor modifications [20]. The livers were perfused in situ via the portal vein with 75 ml of Krebs Ringer Buffer (KRB), followed by 100 ml of KRB containing collagenase at a concentration of 150 units/ml. The flow rate was 10 ml/min. Digested liver was then removed and shaken in Petri dishes with sterile forceps to release the cells into KRB. Isolated cells were collected with a 60 μ m cell filter and low speed centrifugation (450 rpm). Viable hepatocytes were further purified by the isodensity Percoll centrifugation method and plated onto 60-mm plastic dishes at a density of 3×10^5 hepatocytes per dish. The basal culture medium was DMEM supplemented with 10% fetal calf serum and Penicillin/streptomycin. Viability of the hepatocytes was evaluated by the trypan blue exclusion test and the culture medium was renewed 4 hours after initial plating. From the second day on, simvastatin was added at a concentration of 20 μ g/ml of medium. The medium was changed once per day for 3 days. Protein was then isolated from the cultured cells for analysis.

Statistical Analysis

Data are presented as mean values $\pm$ SEM. Statistical evaluations were performed using ANOVA and Student *t*-tests wherever indicated. *P* values less than 0.05 were considered significant and *n* represents the number of animals per group.

RESULTS

Burn induced liver damage and cellular apoptosis

Figure 1 illustrates H&E and TUNEL stained section of livers from burned mice and sham treated controls. Sham-treated mice exhibited normal hepatic structure (Fig. 1A), whereas, mice with burn injuries presented significant liver damage with broad areas of cellular destruction (Fig 1B). TUNEL staining of the liver sections demonstrated occasional apoptosis in the sham treated mice (Fig. 1C). In contrast, a remarkable increase in the number of TUNEL-positive hepatocytes indicated widespread hepatocyte apoptosis in the livers of burned mice (Fig. 1D). The apoptotic cells were present in a diffuse distribution pattern.

Figure 2 illustrates the time course of apoptosis in the livers from burned mice and sham treated controls. Apoptosis was detected within 6 hours after the burn increased over time, peaked at 24 hours post-burn injury and decreased gradually thereafter. The striking apoptosis was apparent throughout the entire course of the 6-day study in thermally injured mice. Two-way ANOVA demonstrated significant main effects of treatment (burn vs. sham): $F_{1, 50}=647.27, p<0.0001$; time after injury: $F_{4, 41}=96.44, p<0.001$ and treatment by time interaction: $F_{4, 41}=80.77, p<0.0001$. In sham treated animals there was no evidence of increased apoptosis over the 6 day observation period. At 12 hours, 1 day, 3 days and 6 days after injury there was increase apoptosis in the livers of burned compared with sham treated

animals ($p<0.05$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively. At 1, 3 and 6 days after injury apoptosis was increased compared to 12 hours after injury ($p<0.001$, $p<0.01$ and $p<0.05$ respectively). Compared to 1 day after injury apoptosis decreased at 3 and 6 days after injury ($p<0.05$). These findings indicate that a full-thickness burn in mice produces remote hepatic injury that is manifested as significant hepatocellular apoptosis.

Effect of burn injury on TNF- α and caspase-3 expression in liver

Figure 3 illustrates the levels of TNF- α and caspase-3 expression in the livers from burned mice and sham treated controls. In the sham treated animals, expressions of TNF- α and caspase-3 were mildly detectable in the liver; however, when the mice were exposed to thermal injury, both TNF- α and caspase-3 expressions were remarkably increased. The time course data demonstrated that both TNF- α and caspase-3 expression gradually increased post-burn injury, peaking at 12 to 24 hours post-burn and gradually decreasing at 3 days post-burn.

Effect of simvastatin on burn-induced hepatic cellular apoptosis

The effect of simvastatin treatment on burn-induced hepatic cellular apoptosis is illustrated in Figure 4. One-way ANOVA demonstrated a highly significant effect of treatment $F_{3,23}=73.06$, $p<0.0001$. In contrast to the untreated and saline-treated mice, simvastatin reduced the apoptosis index significantly ($p<0.001$) at 24 hours after the burn injury, indicating a strong anti-apoptotic property of simvastatin in peripheral burn-induced liver injury.

Effect of simvastatin on burn-induced TNF- α and caspase-3 expression in livers and cultured mouse hepatocytes

Figure 5 illustrates the effect of simvastatin on TNF- α and caspase-3 expression. Compared with saline-treated mice, simvastatin-treated mice showed a large reduction in TNF- α and caspase-3 expression in the liver. Simvastatin treatment decreased burn-induced hepatic cellular apoptosis, and reduced TNF- α and caspase-3 expression. These findings indicate that simvastatin can reduce hepatocyte apoptosis by suppressing the TNF- α /caspase-3 signaling pathway (Fig. 5. A, B).

The effects of simvastatin were further substantiated by an in vitro study in which cultured mouse hepatocytes were treated with simvastatin for 3 days. The results showed that simvastatin decreased both TNF- α and caspase-3 expression in the cultured cells (Fig. 5. C, D).

Effects of TNF- α and caspase-3 inhibitors and simvastatin in burn injury induced hepatic apoptosis

Figure 6 illustrates the effect of TNF- α and caspase-3 inhibitors on hepatic apoptosis in burned mice. One-way ANOVA on the TNF- α data in figure 6. A demonstrated a highly significant effect of treatment: $F_{4,28}=60.37$, $p<0.001$. As expected burn produced a significant increase in apoptosis ($p<0.001$). This effect was partially reversed by treatment with TNF- α inhibitor, inhibitor plus statin ($p<0.01$). One-way ANOVA of the caspase-3 data in figure 6. B demonstrated a highly significant effect of treatment: $F_{4,28}=42.65$, $p<0.001$.

As expected burn produced a significant increase in apoptosis ($p<0.001$). This effect was partially reversed by treatment with caspase-3 inhibitor, inhibitor plus statin ($p<0.01$). Overall, these results indicate that in burned animals, the treatment effects of simvastatin on hepatocytic apoptosis were partially blocked by Pentoxifyline and Ac-DEVD-CHO, suggesting a potential pathway by which simvastatin exerts its anti-apoptotic activity.

Hepatic apoptosis in $\text{TNF-}\alpha$ -/- and caspase-3-/- mice

Figure 7 illustrates the effects of $\text{TNF-}\alpha$ and caspase-3 knockout on burn-induced liver apoptosis. One-way ANOVA on $\text{TNF-}\alpha$ KO's data demonstrated a highly significant effect of treatment: $F_{4, 28}=67.93$, $p<0.0001$. As expected burn produced a significant increase in apoptosis ($p<0.001$). This effect was significantly ($p<0.01$) reduced by all of the treatments. One-way ANOVA on caspase-3 KO's data demonstrated a highly significant effect of treatment: $F_{4, 24}=42.99$, $p<0.0001$. As expected burn produced a significant increase in apoptosis ($p<0.005$). This effect was significantly ($p<0.01$) reduced by all of the treatments. Overall, these results indicate that compared with wild-type mice, $\text{TNF-}\alpha$ -/- and caspase-3-/- mice demonstrated significantly lower levels of apoptosis in the liver after burn injury. Levels of hepatic apoptosis in knockout mice treated with simvastatin were not significantly different from levels of these mice without statin treatment. These findings further indicated that simvastatin reduces apoptosis in burn injured mice, at least partially, via the $\text{TNF-}\alpha$ /caspase-3 pathway.

DISCUSSION

Statins, HMG-CoA reductase inhibitors, are cholesterol-lowering agents that are widely used in the treatment and prevention of atherosclerosis [21, 22]. The present study investigated the effects of simvastatin on hepatocellular apoptosis in response to burn injury in mice, and further evaluated the underlying mechanism through which simvastatin protects the liver.

Burn injury induced apoptosis in mouse liver

The results of the present study indicated that severe burn injury leads to a broad area of cellular destruction in mouse liver. Furthermore, TUNEL staining demonstrated remarkable hepatic apoptosis in the livers of burn-injured mice throughout the study period. These results confirmed that damage to the liver is a direct effect of burn injury and mainly presents as hepatocellular apoptosis. These findings are consistent with previous clinical investigations which have demonstrated that severe burn injury can induce significant hepatic dysfunction [23]. The thermal injury animal model used in the present study accurately represents the phenomena observed in burn patients. Previous studies have also suggested that remote liver injury may be induced by tissue oxidative stress provoked by burn injury and that this stress is closely related to several inflammation-related processes during which many cytokines and chemokines play important roles [24].

Inflammation and hepatic apoptosis

The role of $\text{TNF-}\alpha$ in apoptosis has been well-established. $\text{TNF-}\alpha$ acts as an extrinsic stimulator that triggers cellular apoptosis. TNF receptor-associated death domain (TRADD)

and Fas-associated death domain (FADD) proteins, which leads to activation of caspases, including caspase-3 [25]. TNF- α -induced PI3K activation results in the generation of reactive oxygen species, which then activates caspase-3. The TNF- α /caspase-3 pathway operates in many types of cells, including hepatocytes [26, 27]. In the present study, low levels of TNF- α and caspase-3 expression were detected in the livers of normal mice. However, when mice were exposed to thermal injury, expression of these proteins was significantly increased, indicating that thermal injury leads to activation of the TNF- α /caspase-3 signaling pathway, which is responsible for burn-induced liver injury. This argument is further supported by our findings that use of TNF- α inhibitor and Caspase 3 inhibitor (Fig 6), and depletion of TNF- α or caspase-3 genes in TNF- $\alpha^{-/-}$ and Caspase-3 $^{-/-}$ animals, significantly ameliorated the burn induced hepatic apoptosis (Figure 8). Therefore, these results suggest that TNF- α /Caspase-3 pathway is involved in the burn injury induced hepatocellular apoptosis.

Simvastatin and hepatic apoptosis after thermal Injury, and the TNF- α /Caspase-3 pathway

The results of the present study demonstrated that treatment with simvastatin to burned animals significantly reduced the liver apoptosis index (Figure 4), this was further supported by the in vitro studies that addition of simvastatin into hepatocyte culture medium reduced TNF- α and Caspase-3 expression (Figure 4) indicating its potent anti-apoptotic properties in burn-induced liver injury.

Our studies further revealed the possible mechanism of this protective effect by simvastatin. First, the anti-apoptotic effects of simvastatin was accompanied with a significant reduction of hepatic expressions of TNF- α and caspase-3 in livers from burned animals (Figure 5 A and B). Secondly, TNF- α and caspas-3 inhibitors abrogated the burn injury induced hepatic apoptosis (Figure 6). Thirdly, addition of simvastatin to TNF- α and caspase-3 inhibitors did not show significant further reduction of apoptosis. Fourthly, in TNF- $\alpha^{-/-}$ and caspase-3 $^{-/-}$ animals, burn injury did not cause significant increase of hepatic apoptosis as compared to that in wide type animals (Figure 7). Furthermore, in TNF- $\alpha^{-/-}$ and caspase-3 $^{-/-}$ animals, administration of simvastatin to burned animals did not cause significant reduction in apoptosis. All these findings suggested that the anti-apoptosis effect of simvastatin involves the mechanism of inhibiting TNF- α /Caspase-3 pathway.

The protective role of Simvastatin in burn-induced liver injury and its clinical implication

Hepatic apoptosis has been recognized as part of the pathological changes after burn injury [28, 29]. High apoptotic cell number after burn injury implicates hepatic damage and impairment to its function. Results from the present study strongly suggested a protective role of simvastatin on hepatocyte apoptosis. Clinical reports have demonstrated that, simvastatin decreased the morbidity and mortality of burn patients [12]. Our earlier studies using a burn-sepsis animal model also found that statin treatment reduced total mortality when it started 2h and 4h after the insult [30]. The reduction of mortality is at least partially attributable to that in liver damage [12]. The focus of the present study was to explore burn injury induced hepatic apoptosis, the protective role of simvastatin in reducing the apoptosis and the mechanisms involved in this role. The study was conducted in a 30% full

thickness burn model with low mortality. Therefore the present study did not use mortality as an end point.

In summary, the present study demonstrated that peripheral thermal injury leads to remote hepatocyte apoptosis and that simvastatin treatment can significantly attenuate this process by decreasing activity of the TNF- α /caspase-3 signaling pathway. These novel findings provide a mechanistic platform for understanding how statin treatment reduces morbidity and mortality in burn patients and suggest that the TNF- α /caspase-3 pathway may represent a new target for the development of more effective treatment regimes to protect liver function after burn injury. Further studies will be necessary to completely understand the effects of liver apoptosis on overall survival after thermal injury, and if the protective effects of simvastatin involve other signal transduction pathways of hepatic apoptosis.

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Figure 1. Burn induced remote liver injury

A and B: Representative images of H&E – stained liver sections. A. Sham-treated mice exhibited normal hepatic structure. B. Burn injury produced significant damage to the liver with broad areas of cellular destruction (CV: central vein; PV: portal vein). C and D: Representative histological images of TUNEL-stained liver sections. C. Occasional apoptotic cells were detected in the livers of sham treated mice. D. Increased numbers of TUNEL-positive hepatocytes were observed in the livers of burn injured mice. Apoptotic cells were present in a diffuse distribution pattern. Examples of apoptotic cells are indicated by arrows (5 mice in each time point).

Figure 2. Time course of burn induced hepatocellular apoptosis

Representative histological images of TUNEL stained liver sections. A. Sham-treated mice. B. Mice at one day after burn injury. C. Mice at three days after burn injury. Examples of apoptotic cells are indicated by arrows. D. Percent apoptotic cells at various times after injury. Mean $\pm$ SEM, n=5 animals per group (sham vs. burn: * p <0.05, ** p <0.01, *** p <0.001; within sham group: p >0.05; within burn group: p <0.01 except burn/6hr vs. burn/12hr: p >0.05).

Figure 3. Time course of TNF-α and caspase-3 expression in liver of mice with peripheral burn injury

A. Western blot detection of TNF-α and caspase-3 expression, sham group: n=4, other groups: n=6(the experiment was repeated once). B. Level of TNF-α expression normalized to the expression level of GAPDH. C. Level of caspase-3 expression normalized to the expression level of GAPDH.

Figure 4. Effects of simvastatin on the burn-induced hepatic cellular apoptosis

Representative histological images of TUNEL stained liver sections. A. burned mice without simvastatin treatment. B. burned mice treated with simvastatin. C. Percent apoptotic cells with various treatments. Mean $\pm$ SEM, n=5 animals per group. Burn injury produced significant apoptosis in mouse liver which was significantly reduced by treatment with simvastatin (* p <0.001 vs. sham, and simvastatin/burn; burn vs. burn/saline: p >0.05).

Figure 5. Effects of simvastatin on TNF-α and Caspase-3 expression in livers of mice with burn injury and in cultured mouse hepatocytes

A. Western blots analysis of the effect of simvastatin treatment on TNF-α expression in mouse liver. The graph indicates mean $\pm$ SEM for n=5 animals with each treatment. B. Western blots analysis of the effect of simvastatin treatment on caspase-3 expression in mouse liver. The graph indicates mean $\pm$ SEM for n=5 animals with each treatment. Burn injury increased expression of TNF-α and caspase-3 in mouse liver and this effect was abolished by treatment with simvastatin. C. Western blots analysis of the effect of simvastatin treatment on TNF-α expression in primary cultures of mouse hepatocytes. The experiments were repeated three times with 5 wells per study). D. Western blots analysis of the effect of simvastatin treatment on caspase-3 expression in primary cultures of mouse hepatocytes. The experiments were repeated three times with 5 wells per study). Simvastatin clearly reduced expression of both TNF-α and caspase-3 in these cultured cells.

Figure 6. Effects of TNF- α and caspase-3 inhibitors on hepatic apoptosis in burned mice

A. Effects of the TNF- α inhibitor, Pentoxifyline (50 μ g/kg, IP), on apoptosis. Each value is the mean $\pm$ SEM for n=5 animals with each treatment (* p <0.01 vs. all other groups; ** p <0.01 vs. sham, burn; ** p >0.05: burn/inhibitor vs. burn/inhibitor/statin).

B. Effects of the caspase-3 inhibitor, Ac-DEVD-CHO (50 μ g/kg, IP), on apoptosis. Each value is the mean $\pm$ SEM for n=5 animals with each treatment. Burn injury induced marked hepatocellular apoptosis which was partially reversed by treatment with simvastatin, TNF- α or caspase-3 inhibitors (* p <0.01 vs. all other groups; ** p <0.01 vs. sham, burn; ** p >0.05: burn/inhibitor vs. burn/inhibitor/statin).

Figure 7. Effects of TNF- α and caspase-3 knockout on burn-induced liver apoptosis

A. Effects of TNF- α knockout on apoptosis in mouse liver. Each value is the mean $\pm$ SEM for $n=5$ animals with each treatment (ANOVA: $*p<0.001$; $**p>0.05$). B. Effects of caspase-3 knockout on apoptosis in mouse liver (ANOVA: $*p<0.001$; $**p>0.05$). Each value is the mean $\pm$ SEM for $n=5$ animals with each treatment. Representative histological images of TUNEL stained liver sections are also illustrated with examples of apoptotic cells indicated by arrows. Compared with the wild-type mice, TNF- α -/- and Caspase-3-/- mice demonstrated much lower levels of apoptosis in the liver after burn injury. The levels of apoptosis in the knockout mice treated with simvastatin were not significantly different from levels in knockout mice without statin treatment, indicating that statin might reduce apoptosis in burn injured mice, at least partially, through inhibition of the TNF- α /caspase-3 pathway.