Differential activation of the Stat signaling pathway in the liver after burn injury

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Wang, Shan, Steven E. Wolf, and B. Mark Evers. Differential activation of the Stat signaling pathway in the liver after burn injury. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1153–G1159, 1997.—The liver plays a crucial role in the acute phase response after injury; mechanisms responsible for transducing inflammatory signals to the nucleus to initiate this response are not known. The purpose of this study was to examine the induction of the novel Stat (signal transducer and activator of transcription) pathway in the liver after burn injury. Rats were subjected to either a 60% burn or sham treatment; livers were removed over a time course and extracted for nuclear protein. We found that Stat3, but not Stat5, binding was predominantly increased in the liver after burn injury as assessed by gel mobility and “supershift” analyses. Moreover, Stat3 nuclear protein levels were increased 6- to 14-fold in the livers of burned rats compared with those of sham rats. Stat3 phosphorylation was rapidly induced after burn injury; the subsequent increase of Stat3 binding was completely blocked by preincubation with the antiphosphotyrosine antibody (4G10). We conclude that a differential and early induction of Stat3 binding activity occurs in the liver after burn injury; this induction is mediated by an increase in phosphorylation. These findings suggest an important role for Stat3 in transducing inflammatory signals to the nucleus of liver cells after a systemic burn injury.

signal transduction; hepatic acute phase response

The liver plays a critical role in the inflammatory response that occurs after systemic injury or sepsis; cytokines and other humoral mediators, released by different cell types, induce the hepatic response to acute inflammation (14). This response involves a major “switch” in the synthetic capacity of the liver to the secretion of acute phase proteins that play important roles in, for example, maintaining homeostasis through inactivation of proteases, facilitating wound healing, and scavenging macrophage opsonins (2, 8). This change in hepatic function from a normal to a stress-response state involves a dramatic alteration in the pattern of hepatocyte gene expression (10). Identifying the molecular mechanisms by which inflammatory signals are transduced from the cell surface to the nucleus is critical to our better understanding of the effects of systemic injury on the liver.

The identification of the novel Stat (signal transducer and activator of transcription) signal transduction pathway represents an important advance in our understanding of the mechanisms by which cytokines and other inflammatory mediators regulate various cellular functions (4, 12, 13). The binding of certain cytokines and growth factors to their receptors activates Janus kinases, which then phosphorylate and activate latent cytoplasmic Stat proteins that subsequently translocate to the nucleus and activate target genes (24). The Stat family consists of six distinct but homologous members that are widely expressed in a variety of cell types; tissue specificity to a response is achieved by the preferential activation of specific Stat proteins by various ligands. For example, Stat3 (also called the acute phase response factor) regulates transcription of a subset of acute phase response proteins in response to stimulation by interleukin-6 (IL-6) (32, 35). Stat3 can homodimerize with itself or heterodimerize with Stat1 and bind to a s5-inducible enhancer (SIE) element located in the promoter region of target genes (e.g., c-fos) (19, 28, 35). Stat5, or mammary gland factor, was discovered initially as a protein that binds to DNA sequences essential for lactogenic hormone (25, 29); however, Stat5 appears to regulate certain hepatocyte functions, particularly in response to growth hormone (17). An important problem in understanding the role of this signaling pathway is to explain how selectivity is achieved in various organs (e.g., the liver) with respect to the effects of cytokines and other mediators released after injury.

The purpose of this study was to determine the effect of a systemic injury on the induction of the Stat transduction pathway in the liver. We have utilized a well-characterized burn model (11, 30) to assess changes in Stat1, Stat3, and Stat5 protein binding and steady-state nuclear levels. In addition, the importance of phosphorylation on Stat3 protein binding was ascertained.

MATERIALS AND METHODS

Materials. Restriction, ligation, and other DNA-modifying enzymes were purchased from Promega (Madison, WI) or Stratagene (La Jolla, CA). The protease inhibitors were from Sigma Chemical (St. Louis, MO). Poly(dI/dC) was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ), and radioactive compounds were obtained from DuPont-New England Nuclear (Boston, MA). Autoradiography film (X-Omat) was from Eastman Kodak (Rochester, NY). Oligonucleotides containing a SIE element, which binds both Stat1 and Stat3, a rat β-casein probe, which binds Stat5, and consensus and mutant Stat3 binding sites were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to Stat3 (sc-591) and Stat3 (sc-482) for Western blots and “supershift” studies and goat anti-mouse immunoglobulin G (IgG) (sc-2005) were also from Santa Cruz Biotechnology. Donkey anti-rabbit IgG (NA934) was obtained from Amersham (Arlington Heights, IL). Monoclonal anti-Stat5 (s21520) was from Transduction Laboratories (Lexington, KY), and monoclonal antiphosphotyrosine antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescence (ECL) system for Western immuno blot analysis was from Amersham. All other reagents were of molecular biology grade and...
were obtained from either Sigma Chemical or Amresco (Solon, OH).

Animals and experimental design. Male Fischer 344 rats (276–385 g), purchased from Harlan Sprague Dawley (Indianapolis, IN), were acclimated for at least 1 wk with 12:12-h light-dark cycles in individual wire-bottomed cages and fed standard laboratory chow (FormulaLab Chow, Purina Mills, St. Louis, MO) ad libitum. After acclimation, rats were anesthetized with intraperitoneal pentobarbital sodium (30 mg/kg) and received either a 60% total body surface area (TBSA) third-degree burn or sham treatment, as described previously (11, 30). Briefly, the dorsal and ventral surfaces of the rats were shaved before placing the animals in a mold designed to administer a 30% burn. Rats were submerged in heated water (−98°C) for 10 s on the dorsal surface and 2 s on the ventral surface to produce a 60% TBSA burn. Sham rats were treated identically except they were dipped in water at room temperature.

Liver samples from the three rats at each time point were pooled, and nuclear extracts were prepared from the freshly excised rat livers, according to the method described by Gorski et al. (9), except that the phosphatase inhibitors sodium orthovanadate (1 mM) and sodium fluoride (10 mM) were added to the homogenization and nuclear lysis buffers. In addition, the following protease inhibitors were added to the initial homogenization buffer: chymostatin, pepstatin (each at 2 µg/ml), aprotinin, leupeptin (each at 5 µg/ml), trypsin inhibitor (10 µg/ml), and phenylmethylsulfonyl fluoride (PMSF; 0.1 mM). The final nuclear preparation was dialyzed against NED buffer [25 mM N-2-hydroxyethylpipеразин-N’-2-этилентетрауксусной кислоты (HEPES), pH 7.6, 40 mM KCl, 15 mM PMSF, 1 mM sodium orthovanadate, and 10% glycerol]. The extracts were quick frozen and stored in aliquots at −80°C.

For electrophoretic mobility shift assays (EMSA), liver nuclear protein (10 µg) was preincubated for 10 min at 4°C with 5× binding buffer and 1 µg of poly(dI/dC). The synthetic SIE or Stat5 double-stranded oligonucleotide probes were end-labeled on one strand with [γ-32P]ATP and T4 polynucleotide kinase. EMSA mixtures contained 45,000 counts/min of 32P-end-labeled oligonucleotide and 10 µg of nuclear protein in a final volume of 20 µl of 12.5 mM HEPES (pH 7.9), 100 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.75 mM dithiorthetol, 0.2 mM PMSF, and 1 µg of bovine serum albumin with 1 µg of poly(dI/dC) as nonspecific competitors and were incubated for an additional 20 min at room temperature. Competition binding experiments were performed as described previously (6) by first incubating the competitor fragment, in molar excess, with the nuclear protein and binding buffer for 10 min on ice. The labeled probe was then added, and the incubation was continued for 20 min at room temperature. The reaction mixtures were loaded onto 6% nondenaturing polyacrylamide gels and resolved by electrophoresis in 0.5× Tris borate-EDTA buffer for 2 h at 200 V. For antibody (supershift) studies, 3 µl of antiserum to either Stat1 or Stat3 were added during the preincubation for 1 h at room temperature before the addition of labeled probe. To increase the resolution of the gel shift complexes, the electrophoresis time was increased to 3.5 h, resulting in the elution of unbound probe from the bottom of the gel. To confirm the importance of tyrosine phosphorylation for Stat3 binding, liver nuclear extracts were preincubated for 1 h with 3 µl of the antiphosphotyrosine antibody (4G10) or ice and analyzed by EMSA using the labeled SIE probe. The gels were subsequently dried and autoradiographed at −70°C with an intensifying screen.

Western immunoblot and immunoprecipitation analyses. Western immunoblot analyses were performed as described previously (5). Briefly, protein samples (30 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted to Immobilon P nylon membranes. Filters were incubated overnight at room temperature in blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.05% Tween 20), followed by a 4-h incubation with either rabbit anti-Stat1 (1:500), rabbit anti-Stat3 (1:500), or mouse anti-Stat5 (1:1,000) antibodies. Filters were washed three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG as a secondary antibody (1:1,000) for 1 h. After three final washes, the immune complexes were visualized using ECL detection.

For immunoprecipitation analysis, nuclear extracts (100 µg) were precleared with protein G-Sepharose (50 µl of a 50% suspension) for 1 h at 4°C in immunoprecipitation buffer (total vol, 1 ml). Samples were briefly spun, and the supernatants were transferred to another 50 µl of protein G and then incubated with 3 µl of anti-Stat3 overnight at 4°C with gentle shaking. Samples were centrifuged for 4 min at 14,000 g, and the Sepharose pellets were washed three times with 500 µl of immunoprecipitation buffer. The washed beads were resuspended in 30 µl of 2× sample loading buffer, boiled for 10 min, and centrifuged for 4 min at 14,000 g. The supernatant was run on a 7.5% SDS-PAGE gel, and immunoblots were probed with either anti-Stat3 or antiphosphotyrosine (4G10).

RESULTS

Binding to the SIE element is increased in the liver after burn injury. To examine the possible role of the Stat pathway in the liver after injury, we first assessed binding activity of Stat proteins (1, 3, and 5) by EMSA. An oligonucleotide containing a consensus SIE element from the c-fos promoter (28) was used to determine Stat1 and Stat3 binding. Activated Stat1 and Stat3 proteins bind to the SIE element and can form three distinct gel shift complexes: an upper band (complex A) consisting of Stat3 homodimers, a middle band (complex B) consisting of Stat3 and Stat1 heterodimers, and a lower band (complex C) consisting of Stat1 homodimers (19, 35).

As shown in Fig. 1, an increase in protein binding to the labeled SIE probe (complexes A and B) was noted in livers from burned rats (Fig. 1, lanes 6–9) compared with livers harvested from the sham group (Fig. 1, lanes 2–5). The increase in complex A (Fig. 1, upper band) was noted by 1 h, since earlier time points (i.e., 15, 30, and 45 min) did not demonstrate a significant increase in binding activity (data not shown); binding activity remained elevated for the duration of the experiment (48 h). A smaller increase in binding activity was noted in the sham livers, which is probably related to the stress of anesthesia and manipulation. A lower protein-DNA complex (complex B) was noted 2 h...
after either burn or sham treatment; however, complex B was never as pronounced as complex A. We did not demonstrate a lower third complex (complex C) after burn injury. The specificity of the binding of both complexes was confirmed by inhibition of complex formation using a 200-fold molar excess of the unlabelled SIE probe (Fig. 1, lane 10).

In contrast, although binding to the consensus Stat5 binding site demonstrated minor changes in both the burn and sham groups, the differences between the two groups were not significant (data not shown). Therefore, our findings demonstrate a more pronounced increase in protein binding to the SIE probe in liver extracts from burned rats. Presumably, the induction of protein binding (complex A) noted early after burn injury and persisting for 2 days postburn should be a result of increased binding activity of Stat3 homodimers; however, the exact nature of these complexes (i.e., predominantly Stat1 or Stat3) could not be readily defined by this initial study.

Increases of protein binding to SIE probe predominantly represent an increase in Stat3. To better determine which of the Stat proteins are increased early after burn injury, an additional EMSA was performed using liver nuclear extracts taken 1 h after burn injury (Fig. 2). As previously demonstrated, the entire complex is readily competed with a molar excess of the SIE probe (Fig. 2, lane 3). In addition, the majority of the complex was effectively competed using a molar excess of an oligonucleotide containing the consensus Stat3 binding site (Fig. 2, lane 4). In contrast, an excess oligonucleotide containing the mutated Stat3 binding site did not compete for the complex (Fig. 2, lane 5). Furthermore, addition of antibody to Stat3 (Fig. 2, lane 8), but not Stat1 (Fig. 2, lane 7), produced a supershifted complex. Taken together, these results demonstrate that the increase in protein binding to the SIE probe occurring early (by 1 h) after burn injury was predominantly the result of an increase in Stat3 and not in Stat1.

Nuclear Stat3 protein levels are increased in the livers of burned rats. To next assess whether the differential increases in Stat binding were associated with concomitant increases in nuclear protein levels, Western immunoblot analyses using liver nuclear proteins were performed (Fig. 3). Protein levels of Stat3 were increased 6- to 14-fold in the livers of burned rats (Fig. 3A; lanes 6–9); protein from the livers of rats given LPS was used as a positive control and, as previously shown (17, 21), Stat3 levels were increased (Fig. 3A, lane 10). Similar to our gel shift analyses, sham treatment resulted in minor increases in Stat3 levels, which were not elevated to the degree noted after burn injury (Fig. 3A, lanes 2–5). Stat1 protein levels were increased in the livers of burned rats, with a maximal elevation occurring at 24 h after burn (Fig. 3B, lane 8); no increases were noted with sham treatment (Fig. 3B, lanes 2–5). Similar to previous reports (17, 21), Stat1 was not increased in the liver after LPS administration (Fig. 3B, lane 10). Finally, consistent
with our gel shift results, there were no differences in Stat5 protein levels in the livers of the burned animals compared with those of sham-treated animals (data not shown).

Collectively, these findings confirm the differential induction pattern of the Stat proteins as demonstrated by increases in both Stat3 binding and nuclear protein levels in the liver after burn injury. Stat1, although increased, was not elevated to the same degree as Stat3. Moreover, Stat5 levels in the liver were not specifically altered by burn injury.

Activation of Stat3 after burn injury occurs by tyrosine phosphorylation. Stat proteins are activated by tyrosine phosphorylation in response to various inflammatory mediators (e.g., interferon, IL-6, and LPS) (17, 19, 35). We next determined whether burn injury resulted in Stat3 phosphorylation using liver nuclear extracts immunoprecipitated with anti-Stat3 antibody and the blot probed with an antiphosphotyrosine antibody (4G10) (Fig. 4A). Burn injury induced tyrosine phosphorylation (PY-Stat3) by 1 h, with maximal induction occurring at 2 h (Fig. 4A, lanes 6 and 7), and, as
expected, treatment with LPS (positive control) induced Stat3 phosphorylation (Fig. 4A, lane 10). No increase of phosphorylation was noted with sham treatment. The blot was stripped and reprobed with anti-Stat3 antibody; relatively equal amounts of Stat3 were detected in each lane of the immunoprecipitated samples after burn injury (Fig. 4B). Consistent with our previous results by Western blot (Fig. 3A), Stat3 levels were minimally increased in the livers of sham-treated animals.

Finally, to confirm that tyrosine phosphorylation is required for the induction of Stat3 protein binding occurring after a burn, liver nuclear extracts from 2-h postburn and LPS-treated rats were preincubated with 4G10 and analyzed by EMSA using the labeled SIE probe (Fig. 5). Preincubation with 4G10 completely blocked binding to the SIE probe in liver extracts from rats after a burn (Fig. 5, lane 4) or injected with LPS (data not shown). In contrast, incubation with preimmune IgG had no effect on protein binding (Fig. 5, lane 3). Taken together, these results strongly suggest that the increase in Stat3 protein binding after burn injury is mediated by the induction of phosphorylation.

DISCUSSION

In our present study, we have utilized a well-described and standardized rat burn model (11, 30) to analyze the Stat signal transduction pathway in the liver after a systemic injury. Our study specifically focused on Stat1, Stat3, and Stat5 since they are present in several cell types (including hepatocytes), are activated by multiple ligands, and appear to play various functional roles in the liver in response to mitogenic or inflammatory signals (3, 18, 20, 21, 26, 27, 31). In contrast, the other Stat proteins (Stat2, Stat4, and Stat6) are more highly specialized, are only activated by a select and limited number of ligands, and participate more in immune function (24).

We demonstrated a marked and early (by 1 h) induction of Stat3 protein levels and actual binding activity in the liver after burn injury; Stat1 levels were elevated, but not nearly to the level of Stat3. This early activation and nuclear translocation of Stat3 suggests an important role for this factor in the early response of the liver to a systemic injury. Consistent with our findings, levels of Stat3 have been shown to be increased in the liver during periods of rapid proliferation or as a response to administration of an inflammatory agent. For example, Stat3 binding activity and nuclear protein levels were significantly increased in the remnant liver after partial hepatectomy in the rat (3, 27). Furthermore, Ripperger et al. (18) demonstrated an increase in Stat3 binding activity in the rat liver after intraperitoneal injection of complete Freund's adjuvant, a potent stimulator of the hepatic acute phase response. Therefore, similar to our present study utilizing a systemic burn injury, an early and prolonged induction of Stat3 occurs in the liver in response to both inflammatory and proliferative signals. Taken together, these findings suggest that Stat3 may activate
The Stat proteins are latent transcription factors that appear to be activated by tyrosine phosphorylation (15, 19, 20, 31). After phosphorylation, the Stat proteins homo- or heterodimerize, translocate to the nucleus, and bind to the regulatory elements of target genes. We have shown that an increase in Stat3 phosphorylation occurs by 1 h after burn injury. This increase in protein phosphorylation is critical to the subsequent increase of Stat3 binding activity since binding to the labeled SI oligonucleotide was completely blocked by preincubation with the antiphosphotyrosine antibody (4G10). Therefore, consistent with other reports utilizing different models of inflammation (3, 17), the induction of Stat3 after burn injury appears to be mediated by an increase in phosphorylation.

The cellular mediators that produce activation of Stat3 after burn injury were not specifically assessed in this study. A number of ligands including epidermal growth factor (EGF), platelet-derived growth factor, colony stimulating factor-1, and IL-6 can activate both Stat3 and Stat1. IL-6 appears to induce Stat3 more strongly than Stat1 (24) and, furthermore, IL-6 is increased in both patients and rats after burn injury (1, 16). Therefore, IL-6 may be one of the cytokines contributing to the more pronounced induction of Stat3. This remarkable specificity in Stat activation in response to burn injury is quite similar to the response after treatment with LPS that we and also Ram et al. (17) and Ruff-Jamison et al. (21) have noted. That is, treatment of rats with LPS, which stimulates release of multiple cytokines in vivo, activated only Stat3, and not Stat1, in the rat liver. Future studies are required to better delineate the specific cellular factors, released after burn injury, that result in this preferential induction of Stat3.

In addition to Stat3 and Stat1, we also evaluated the Stat5 protein and found nonspecific increases in the livers from sham-treated rats (anesthetized without burn), which were no different from the small elevations noted after burn injury. The absence of a clear induction of Stat5 after burn injury was somewhat surprising since Stat5 has been shown to play a role in the hepatic response to several cellular mediators, including growth hormone, prolactin, IL-6, and epidermal growth factor (20, 31); however, we speculate that, similar to Stat1, the specificity for the ultimate response of the liver to a particular inflammatory stimulus may be achieved by differences in the activation of various members of the Stat pathway. Therefore, our findings as well as those in other studies (3, 18, 21, 27, 32, 35) suggest that the acute phase response (which is presumably IL-6-mediated) favors activation of Stat3.

In conclusion, our findings indicate that burn injury induces a differential activation of the Stat proteins. Stat3 levels and binding activity are dramatically increased in the liver after burn; Stat1 was also elevated, but to a lesser degree than Stat3. These results suggest that Stat3 may play an important role in the early response to injury exhibited by the liver. Stat3 may be induced by a number of inflammatory mediators that ultimately culminate in the downstream synthesis of a variety of acute phase proteins. An in-depth analysis of the intracellular mechanisms regulating these early events will lead to a better understanding of the hepatic responses that occur after injury.

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REFERENCES


STAT PROTEINS IN THE LIVER


