Tissue hypoxia is a common sequel of trauma-hemorrhage but can occur even without blood loss under hypoxic conditions. Although hypoxia is known to upregulate Kupffer cells (KC) to release cytokines, the precise mechanism of release remains unknown. We hypothesized that Src family kinases play a role in mediating KC mitogen-activated protein kinase (MAPK) signaling and their cytokine production after hypoxia. Male C3H/HeN mice received either Src inhibitor PP1 (1.5 mg/kg body wt) or vehicle 1 h before hypoxia. KCs were isolated 1 h after hypoxia, lysed, and immunoblotted with antibodies to Src, p38, ERK1/2, or JNK proteins. In addition, KCs were cultured to measure interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) production. Hypoxia produced a significant increase in KC Src and MAPK (p38, ERK, JNK) activity compared with normoxic controls. This was associated with an increase in IL-6 and MCP-1 production. Treatment with PP1 abolished the increase in KC Src activation as well as p38 activity. However, PP1 did not prevent the increase in KC ERK1/2 or JNK phosphorylation. Furthermore, administration of PP1 prevented the hypoxia-induced increase in IL-6 but not MCP-1 release by KC. Additional in vitro results suggest that p38 but not ERK1/2 or JNK are critical for KC IL-6 production. In contrast, the production of MCP-1 by KC was found to be independent of MAPK. Thus hypoxia increases KC IL-6 production. In contrast, the production of MCP-1 by KC was found to be independent of MAPK. Thus hypoxia increases KC IL-6 production. In contrast, the production of MCP-1 by KC was found to be independent of MAPK. Thus hypoxia increases KC IL-6 production.

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this study (n = 6 per group). The mice were allowed to acclimatize in the animal facility for at least 1 wk before experimentation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham, and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Marine model of hypoxia. The hypoxia model used in this experiment was described in detail by Ertel et al. (17). Briefly, mice were fasted overnight but allowed water ad libitum before the procedure. The animals were placed in two plastic chambers (20 × 10 × 8 cm), each with an inlet and outlet, through which the hypoxic gas mixture or room air flowed. Hypoxia was induced by flushing one of the chambers with a gas mixture of 95% N2-5% O2 at a flow rate of 5 l/min for 60 min. At the same time, control (normoxic) animals were kept in the second chamber, which was flushed with room air for 60 min. The animals were constantly monitored during this period, and no immediate or late mortality was observed with this hypoxia model. Previous studies (17, 33) using this murine hypoxia model have shown that in male mice, arterial Po2 decreased to ~40 mmHg within 10 min of hypoxia, remained at that level throughout the duration of hypoxia, but returned to a baseline of 120 mmHg within minutes after the end of hypoxia. The mice were symptomatic for hypoxia, displaying rapid shallow breathing and minimal physical activity.

In a separate experiment, animals received an intraperitoneal injection of PP1 (Biomol, Plymouth Meeting, PA) or PP2 (Calbiochem, La Jolla, CA) each of which was dissolved in DMSO and further diluted in phosphate-buffered saline. The dosage used for both compounds was 1.5 mg/kg body wt and each was administered 1 h before hypoxia. An intraperitoneal injection in mice of appropriately diluted DMSO in PBS vehicle (GIBCO Invitrogen, Carlsbad, CA) served as the vehicle control.

Harvesting procedures. The animals were anesthetized with isoflu- rane and euthanized 1 h after normoxia or hypoxia. Blood was obtained via cardiac puncture using a syringe coated with EDTA (Sigma, St. Louis, MO). Blood was centrifuged (10,000 rpm, 10 min, 4°C) and the plasma stored at −80°C until analyzed. The liver was flushed to remove cellular blood components by a retrograde perfusion with 20 ml of ice-cold Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY) through the portal vein. This was imme- diately followed by perfusion with 15 ml of 0.05% collagenase class IV (Worthington Biochemical, Lakewood, NJ) in HBSS at 37°C. The liver was removed en bloc and transferred to a petri dish containing warm enzyme HBSS.

Preparation of Kupffer cells. Kupffer cells were isolated as described previously, with some modification (40). In brief, the perfused liver was minced finely, incubated at 37°C for 15 min, and passed through a sterile 150-mesh stainless steel screen into a beaker containing 10 ml of cold HBSS and 10% FBS (low endotoxin, GIBCO). The hepatocytes were then removed by low-speed centrifugation at 50 g for 3 min, and the residual cell suspension was centrifuged twice at 400 g, 4°C for 10 min. The supernatant was discarded and the cell pellet was resuspended in Complete medium [Williams E medium containing 10% heat-inactivated FBS and 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml gentamicin (all from GIBCO)]. The cell suspension was then layered over 14% Histodenz (Sigma-Aldrich) in HBSS and centrifuged at 3,000 g, 4°C, for 45 min to separate the Kupffer cells (which form a band at the Histodenz cushion interface) from the remaining parenchymal cells in the pellet. After the nonparenchymal cells were removed from the interface with a Pasteur pipette, the cells were washed twice by centrifugation (800 g, 10 min, 4°C) in complete medium and adjusted to a final concentra- tion of 5 × 10⁶/ml in complete medium. One hundred microliters of this suspension were then transferred to each well of a 96-well tissue culture plate (6.4 mm well diameter) and the cells were allowed to adhere to the plastic surface at 37°C, 5% CO2, and 95% humidity for 2 h. Nonadherent and nonviable cells were then removed by three repeated washings. This protocol provides adherent cells that are 96% positive by nonspecific esterase staining and that exhibit typical macrophage morphology (48).

Cell extract preparation. For protein immunoblotting, the adherent Kupffer cells were lysed with 100 μl/5 × 10⁶ cells of ice-cold lysis buffer (150 mM NaCl, 1 mM MgCl2, 50 mM HEPES, 1 mM EDTA, 0.5% Triton X-100, 10% glycerol, 200 μM soybean orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 200 μM phenylmethylsulfonyl fluoride, 520 μM AEBSF, consisting of 400 μM aprotinin, 10 μM leupeptin, 20 μM bestatin, 7.5 μM pepstatin A, 7 μM E-640 for 60 min at 4°C. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were stored at −80°C until further analysis.

Western blot analysis. Cell extracts (8 μg protein per lane) were electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gels, as described previously (34). Proteins were electroblotted to Immobilon-P membranes (GE Healthcare, Waukesha, WI) using a semidy transblot system (Bio-Rad, Hercules, CA) at 11 V for 45 min. Blots were then blocked for 2 h at room temperature with 5% nonfat dry milk in 50 mM Tris·HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20 (TBST). After two 5-min washes with TBST, membranes were incubated overnight at 4°C with antibodies to phosho-Src Family (Tyr416), phospho-p38 MAPK (Thr180/Tyr182), phospho-p44/42 (phospho-ERK1/2) MAPK (Thr202/Tyr204), phosho-JNK MAPK (Thr183/Tyr185), nonphospho-Src, nonphospho-p38 MAPK, nonphospho-p44/42 (ERK1/2) MAPK, and nonphospho-JNK MAPK (Cell Signaling Technology, Beverly, MA), diluted 1:1,000 in TBST containing 0.5% nonfat dry milk. After subsequent washes with TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Bio-Rad), diluted 1:5,000.

Membranes were developed with the use of an enhanced chemi- luminescence reagent (GE Healthcare), followed by exposure to Biomax light film (Kodak, Rochester, NY). After the film was developed, Western blots were evaluated by densitometric analysis using Ambis optical imaging system (Ambis Systems, San Diego, CA).

Cytokine analysis. The adherent cells were cultured to assess their cytokine production capacity. After 24 h in culture, cell-free supernatants were harvested. The concentrations of IL-6, IL-10, and MCP-1 in these supernatants were measured by commercially available CBA Mouse Inflammation Kits (BD Pharmingen, San Diego, CA), according to the manufacturer’s instructions. Briefly, 50 μl of mixed capture beads were incubated with 50 μl of supernatant and 50 μl of PE detection reagent for 2 h at room temperature. The immunocomplexes were then washed and analyzed using the LSR II flow cytometer (BD Biosciences, Mountain View, CA). Data processing was carried out using the accompanying FACSDiva and BD CBA software.

In a separate experiment, Kupffer cells were obtained from healthy animals and treated with MAPK inhibitors to determine the role of p38, ERK1/2, and JNK in Kupffer cell IL-6 and MCP-1 production. Briefly, the adherent cells were treated with selective inhibitors: a selective and cell-permeable inhibitor of MAPKKK, which acts by inhibiting the activation of ERK1/2 and subsequent phosphorylation of MAPK (PD-98059; 20 μM; IC50 = 2 μM), a highly specific and cell-permeable inhibitor of p38 MAPK (SB-203580; 5 μM; IC50 = 600 nM), and a potent, cell-permeable, selective, and reversible inhibitor of c-Jun NH2-terminal kinase (JNK) (JNK inhibitor II/SP-600125; 20 μM; IC50 = 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3) (all from Calbiochem). Cells were then cultured for 24 h, the cell-free culture supernatants harvested and frozen at −80°C until further analysis. A minimum of two wells were set for each condition.

Statistical analysis. Data are expressed as means ± SE and compar- isons were analyzed using t-test for single comparisons or ANOVA for multiple comparisons. A P value ≤0.05 was considered to be statistically significant for all analyses.
RESULTS

Hypoxia increases Kupffer cell cytokine production as well as Src and MAPK signaling. To investigate the effects of hypoxia on Kupffer cell inflammatory cytokine production, Kupffer cells were cultured for 24 h and the culture supernatants analyzed. We found a significant increase in Kupffer cell IL-6 production (Fig. 1A) after hypoxia compared with normoxic controls. Furthermore, MCP-1 production (Fig. 1B) was increased by >75% in post-hypoxic Kupffer cells compared with those obtained from normoxic mice.

The phosphorylation of both Src family kinases and MAPK by immunoblot was also measured (Fig. 2). Src phosphorylation was found to be significantly increased in Kupffer cells from hypoxic animals compared with normoxic mice. Likewise, the phosphorylation of MAPK (p38, ERK, and JNK) was significantly increased in the hypoxic groups compared with normoxic controls. No significant change in Src and MAPK protein expression was observed following hypoxia.

Effect of PP1 and PP2 treatment on Kupffer cell Src phosphorylation. Mice were treated intraperitoneally with PP1 or PP2 inhibitor before hypoxia to investigate the effects of these compounds on Src phosphorylation and thereby prove their effectiveness as Src inhibitors. Kupffer cell lysates obtained from mice pretreated with vehicle, PP1, or PP2, 1 h before hypoxia were blotted with phospho-Src family (Tyr416) antibody. Levels of phosphorylated Src increased 2.5-fold after hypoxia (Fig. 3). Administration of both PP1 and PP2 compound prevented the increase in Src phosphorylation after hypoxia compared with normoxic controls. However, PP2 appears to be less efficient in preventing the upregulation of phospho-Src after hypoxia than PP1. The reason for differential effects of PP1 and PP2 remains to be established. It may be because PP1 inhibits Src family members p56 (Lck), p59 (Fyn), c-Src, and Hck, whereas PP2 significantly affects only p56 (Lck), p59 (Fyn), and Hck kinases and therefore has a limited inhibition spectra. In view of these findings, PP1 inhibitor was chosen for all further experiments.

Src inhibition differentially influences MAPK signaling. To elucidate whether the blockade of Src family kinases also influences MAPK signaling, a group of mice was treated with Fig. 1. Kupffer cell interleukin (IL)-6 (A) and monocyte chemoattractant protein-1 (MCP-1) (B) production in Kupffer cells following hypoxia (HP). Kupffer cells were isolated (5 × 10⁶ cells/ml) and cultured for 24 h in a 96-well plate at 37°C and 5% CO₂. Supernatants from 6 animals were analyzed in each group with the use of a cytometric bead array. Cytokine concentrations are shown as means ± SE from 6 animals per group. *P < 0.05, compared with normoxic control.

![Graph A: IL-6 production](image1.png)

![Graph B: MCP-1 production](image2.png)

![Graph 3: Src phosphorylation](image3.png)

![Graph 4: PP1 and PP2 treatment](image4.png)
Src inhibitor PP1. Kupffer cell lysates from these animals were immunoblotted with antibodies for p38, ERK1/2, and JNK. Hypoxia induced a significant increase in phosphorylated p38 levels in Kupffer cells (Fig. 4A). However, administration of PP1 before hypoxia resulted in a significant decrease in p38 levels compared with those in Kupffer cells obtained from vehicle-treated mice. Interestingly, the hypoxia-induced increase in the phosphorylation of ERK1/2 was not influenced by Src inhibition with PP1 compound (Fig. 4B). Although there was a tendency of a decrease (~10%) in hypoxia-mediated JNK phosphorylation in Kupffer cells, this decrease was not found to be significantly different from Kupffer cells of normoxic mice (Fig. 4C). These results indicate that the phosphorylation of p38 but not ERK or JNK MAPK is regulated through a Src-dependent pathway.

**Effects of PP1 treatment on Kupffer cell cytokine productive capacity.** We next examined the effect of PP1 on Kupffer cell IL-6 and MCP-1 production following hypoxia. There was a significant increase in Kupffer cell IL-6 and MCP-1 production as a result of hypoxia (Fig. 5). However, treatment of mice with Src family kinase inhibitor PP1 led to a significant decrease in IL-6 levels (Fig. 5A). In contrast, the blockade of Src family kinases had no effect on the production of the chemokine MCP-1 (Fig. 5B).

In addition, the anti-inflammatory cytokine IL-10 was measured in these supernatants. There were no detectable IL-10 levels in any group (data not shown). These findings suggest that Src plays a role in IL-6 regulation in Kupffer cells whereas the production of MCP-1 is likely to be independent from Src signaling. To further delineate the mechanism of IL-6 and MCP-1 regulation in Kupffer cells, we used inhibitors of MAPK which may be downstream to Src.

**Effects of MAPK on Kupffer cell cytokine production capacity.** These experiments were performed to determine the role of p38, ERK1/2, and JNK in mediating the increased Kupffer cell IL-6 and MCP-1 production with the use of their respective inhibitors. The in vitro treatment of Kupffer cells from healthy animals with the specific p38 inhibitor SB-203580 resulted in a significant decrease in the production of IL-6 compared with untreated cells (Fig. 6A). However, ERK inhibitor PD-98059 or JNK inhibitor showed no effect on the constitutive Kupffer cell IL-6 production. In contrast, although there was a tendency of a decrease in Kupffer cell MCP-1 production following inhibition with both p38 and JNK (Fig. 6B), this decrease was not found to be significantly different from that observed in untreated cells. Furthermore, treatment of Kupffer cells with ERK1/2 inhibitor PD-98059 did not influence the MCP-1 production. These results collectively indicate that Src-mediated p38 upregulation plays a role in Kupffer cell IL-6 production after hypoxia.

**DISCUSSION**

We have demonstrated in this study that hypoxia leads to a significant increase in Kupffer cell p38, ERK1/2, JNK, and Src levels. This increase in MAPK and Src is accompanied with an increase in IL-6 and MCP-1 production in Kupffer cells after hypoxia compared with normoxic controls. Selective inhibition of Src family kinases by treatment of animals with PP1 inhibitor 1 h before hypoxia prevented the increase in p38 phosphorylation. In addition, our findings suggest that Src family kinases are not likely to be critical for ERK1/2 or JNK activation. Treatment of animals with PP1 prevented the hypoxia-induced increase in IL-6 by Kupffer cells. In contrast, Src family kinases appear to have no effect on Kupffer cell
MCP-1 productive capacity. Furthermore, we have shown that Kupffer cell IL-6 production after hypoxia is mediated via p38 but not ERK 1/2 or JNK MAPK pathways. These results collectively suggest that Kupffer cell IL-6 production after hypoxia in male mice involves the Src-mediated activation of p38 mitogen-activated protein kinase through a Src-dependent pathway.

The Kupffer cells are considered to be a critical immune cell population that plays an important role in response to endotoxin (5), burns (15), sepsis (14), and hypoxia (17). Once activated, they serve as a major source of systemic levels of IL-6 (39) and MCP-1 (F. Hildebrand, W. J. Hubbard, M. A. Choudhry, H.-C. Pape, and I. H. Chaudry, unpublished observations). While IL-6 is well known to mediate further proinflammatory responses, MCP-1 plays an important role in the recruitment and activation of leukocytes as well as mediating Th2 responses (19). Studies (18) have shown that MCP-1 is integrated in a complex network of chemokines and the regulation of MIP-1α, MIP-1β, RANTES (regulated on activation of normal T cells), and MIP-2, thus regulating chemotaxis during inflammation in various tissues. Eukaryotic cells regulate various functions via the posttranslational mechanism of protein phosphorylation. This mechanism plays a critical role in regulating various cellular activities including gene expression, cell differentiation, and cell proliferation. However, it is important to note that protein phosphorylation is regulated by many factors, and activation at the single protein level is not necessarily controlled within a single pathway. Furthermore, cross-talk between different signaling cascades is often observed leading to a common effect in cellular response. Although MAPK have been implicated in various injury models of innate immune response, little information has been available about how other signaling cascades may influence their activation in response to a traumatic or hypoxic insult.

Mitogen-activated protein kinases are largely involved in eukaryotic cell signaling (7, 42). Three isoforms, p38, ERK1/2, and JNK, are shown to play a major role in the regulation of cellular responses following their activation with growth factors, osmotic shock, and UV light by inflammatory cytokines. Since 1994, when Han et al. (22) first described the activation of p38 MAPK by LPS in murine macrophages, the role of MAPK signaling pathways in the synthesis of inflammatory cytokines by macrophages has been studied in considerable detail. Previous studies (3, 35, 36) have shown that p38, ERK1/2 and JNK MAPK mediate the response of macrophages as well as T cells to traumatic injury. Furthermore, p38 activation is a critical aspect of Kupffer cell activation following burns, trauma (10), or LPS challenge. In addition, the production of TNF-α by Kupffer cells in response to acute pancreatitis...
has been linked to increased activity of p38, ERK 1/2, and JNK MAPK (38). In the present study, we found that p38 plays a predominant role in Kupffer cell IL-6 production in response to hypoxia. In contrast, the production of MCP-1 was found to be independent of p38. Other studies (12) suggest that ERK1/2 is more critical in regulating Kupffer cell IL-6 production compared with p38. The difference between these studies is likely a result of different animal models and different stimuli used but may also reflect the differential regulatory pathways of Kupffer cells following various types of injury.

Src family tyrosine kinases have been implicated in many signaling pathways in immune cells such as growth, differentiation, and gene transcription (44). Their activity is regulated by tyrosine phosphorylation at two sites with opposing effects. While phosphorylation of Tyr527 renders the enzyme less active, Tyr416 phosphorylation upregulates enzyme activity (21). It is also known that hypoxia can activate c-Src leading to increased expression of vascular endothelial growth factor expression (37). Using anti phospho-Src (Tyr416), we examined Src family kinase activation in Kupffer cells from male mice following hypoxia. Our results showed that hypoxia resulted in increased Src activity. Treatment of the animals with Src inhibitor PP1 prevented the increase in Src phosphorylation as well as the subsequent increase in IL-6 production by Kupffer cells following hypoxia. Thus it is likely that Src tyrosine kinases play a role in mediating Kupffer cell response to hypoxia. We recognize that Src is one of several members of Src family tyrosine kinases and the antibodies to Src protein that we have used in this study may cross-react with other Src family members. Thus the possibility of an involvement of other Src family members in Kupffer cell IL-6 production has not been ruled out and this remains to be determined.

Protein phosphorylation within signaling cascades of innate immunity is known to be an early event in response to various stimuli. Src family members have been shown to be activated as rapidly as 1 min after stimulation and their phosphorylation peaks returned to baseline after 10 min (8). Furthermore, hypoxia leads to decreased organ blood flow and oxygen supply, thus interfering with the bioavailability of any administered pharmaceutical as well as its hepatic and renal clearance from the blood. In view of this, we used an in vivo pretreatment regimen with Src family kinase inhibitor PP1 to achieve sufficient inhibitor concentrations at the cellular level before the hypoxic insult to prevent any Src signaling.

Previous studies have linked MAPK as downstream targets to Src signaling (9). Moreover, Khadaroo et al. (30, 31) reported that the priming of macrophages in response to hemorrhagic shock is mediated through activation of p38 MAPK by an Src-dependent pathway. Consistent with these findings, our results suggest that Kupffer cell p38 activation following hypoxia is dependent on the activation of Src kinases. In contrast, other MAPKs, such as ERK1/2 and JNK were not affected by Src inhibition, indicating that ERK and JNK are not regulated by Src after hypoxia. Moreover, recent reports provide evidence that Src family kinases are likely to play a role in various signaling pathways of innate immunity. In light of this, Aki et al. (1) showed that TLR signaling in macrophages is modulated by COOH-terminal Src kinase, an enzyme regulating Src kinase activity.

To further elucidate the role of MAPK on Kupffer cell cytokine production, additional in vitro studies were performed. The results from these experiments confirmed that p38 is critical to Kupffer cell IL-6 production. In addition, we also examined the effect of ERK1/2 and JNK signaling on Kupffer cell IL-6 and MCP-1 production and found that ERK1/2 inhibitor PD-98059 or JNK inhibitor had no effect on IL-6 levels. This finding is in accordance with recent literature, reporting IL-6 regulation by p38 in various tissues (45). In contrast, MCP-1 production appeared to be independent from MAPK (p38, ERK, and JNK) signaling in the present study. Studies have also revealed controversial findings regarding the regulation of specific cytokines and chemokines by MAPK. Although p38 MAPK decreased MCP-1 levels in human colon epithelial cells (32), Kato et al. (27) have shown that MCP-1 production in murine peritoneal mesothelial cells does not require MAPK activation. The reason for the discrepancy between our findings and those of the above-mentioned studies is unclear.

Although we have not pinpointed the precise mechanism by which hypoxia produces an increase in Src kinase activity, the findings from previous studies suggest that several factors, including alterations in intracellular ATP levels and the release of lactic acid, may contribute to an increased proinflammatory cytokine production following hypoxia. These cytokines consecutively serve as a motor of inflammation under such conditions.

In summary, our study demonstrates that activation of Src kinases following hypoxia results in increased p38 activation and thus leads to an increase in IL-6 production in Kupffer cells from male mice. Although hypoxia also increased MCP-1 production by Kupffer cells, we found that these differences in MCP-1 are independent of Src. Nonetheless, further studies are required to delineate the mechanism of increased MCP-1 following hypoxia. Further studies are also needed to delineate whether Src influences pre- or posttranslational events in the regulation of Kupffer cell IL-6 production. We believe that insight into these signaling pathways after hypoxia is critical for developing pharmacological interventions, as well as for the potential to understand differences in host responses.

GRANTS

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SRC KINASES REGULATE p38-MEDIATED IL-6 PRODUCTION


