Hepatic glutamine transporter activation in burn injury: role of amino acids and phosphatidylinositol-3-kinase

TIMOTHY M. PAWLIK, RÜDIGER LOHMANN, WILEY W. SOUBA, AND BARRIE P. BODE
Surgical Oncology Research Laboratories, Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114-2696

Pawlik, Timothy M., Rüdiger Lohmann, Wiley W. Souba, and Barrie P. Bode. Hepatic glutamine transporter activation in burn injury: role of amino acids and phosphatidylinositol-3-kinase. Am J Physiol Gastrointest Liver Physiol 278: G532–G541, 2000.—Burn injury elicits a marked, sustained hypermetabolic state in patients characterized by accelerated hepatic amino acid metabolism and negative nitrogen balance. The transport of glutamine, a key substrate in gluconeogenesis and ureagenesis, was examined in hepatocytes isolated from the livers of rats after a 20% total burn surface area full-thickness scald injury. A latent and profound twoto threefold increase in glutamine transporter system N activity was first observed after 48 h in hepatocytes from injured rats compared with controls, persisted for 9 days, and waned toward control values after 18 days, corresponding with convalescence. Further studies showed that the profound increase was fully attributable to rapid posttranslational transporter activation by amino acid-induced cell swelling and that this form of regulation may be elicited in part by glucagon. The phosphatidylinositol-3-kinase (PI3K) inhibitors wortmannin and LY-294002 each significantly attenuated transporter stimulation by amino acids. The data suggest that PI3K-dependent system N activation by amino acids may play an important role in fueling accelerated hepatic nitrogen metabolism after burn injury.

liver; glucagon; cell volume; signal transduction

After an initial “ebb phase” lasting 24–36 h, major burn injury elicits a prolonged and pronounced period of hypermetabolism and catabolism (“flow phase”) (36). This catabolic flow phase is characterized by increased glucose and oxidative metabolism, hepatic urea synthesis, gluconeogenesis, net nitrogen loss, muscle proteolysis, and subsequent efflux of amino acids, primarily glutamine and alanine (8). As the primary center of glucose and ammonia homeostasis in the body, the liver has been shown to display marked changes in metabolism after burn injury, including accelerated amino acid flux through gluconeogenic (9) and urea synthetic (45) pathways. The glutamine released from skeletal muscle after burn plays a key role in accelerated hepatic metabolism, serving as a substrate in both gluconeogenesis (33) and ureagenesis (16). As part of the well-characterized “intercellular glutamine cycle” (19) along the liver acinus, glutamine is hydrolyzed to glutamate and ammonia via hepatic glutaminase in the large population (>90%) of hepatocytes containing urea cycle enzymes. By virtue of its matrix location, glutaminase acts as an intramitochondrial ammonia amplification system, helping to efficiently drive the low-affinity rate-limiting urea cycle enzyme carbamoyl phosphate synthetase I (31). Therefore, flux through glutaminase plays a critical role after burn injury, because it is estimated that 80–90% of urinary nitrogen loss in burn patients occurs as urea (36). During accelerated metabolism, the transport of glutamine across the hepatocyte plasma membrane has been shown to constitute a rate-limiting step in its hydrolysis via glutaminase (22, 23, 29), an observation that served as the basis for these studies.

The uptake of glutamine across the plasma membrane of hepatocytes occurs primarily via a Na+-dependent amino acid transporter termed system N (6, 25) for its selectivity for glutamine, histidine, and asparagine only (amino acids bearing nitrogenous side chains). Previous studies from our laboratory (27) showed that burn injury stimulated glutamine transport rates, as measured in hepatic plasma membrane vesicles (HPMV) isolated from liver homogenates. Maximum transport stimulation was proportional to the size of the injury but was transient (peaking at 24 h but waning by 72 h) and mirrored the onset and rectification of burn-induced liver damage. These temporal effects at the plasma membrane level, however, were inconsistent with the advent of sustained hypermetabolism beginning 48 h after burn. This raised the possibility that other forms of transporter regulation may be necessary in vivo to support the markedly accelerated hepatic nitrogen metabolism resulting from this trauma. To address this issue, we chose to study isolated hepatocytes, which allow a more comprehensive assessment of burn-influenced transporter physiology, in which the effects of transporter influences lost in subcellular fractionation such as transmembrane electrical potentials, intracellular amino acid effects, and signal transduction pathways can be observed. In light of the importance of glutamine transport in supporting hepatic ureagenesis and gluconeogenesis, the studies presented here were undertaken to examine the impact of burn injury on system N activity in hepatocytes and to investigate potential signal transduction pathways that may participate in its regulation after this trauma.

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**MATERIALS AND METHODS**

L-[\(^{3}H\)]glutamine was obtained from DuPont NEN (Boston, MA). Collagenase was from Boehringer Mannheim (Indianapolis, IN), and chemicals, perfusion media [Calcium-free minimal essential medium for suspension cultures (S-MEM)], glucagon, and unlabeled amino acids were from Sigma (St. Louis, MO). Tissue culture medium (RPMI 1640) and Select-Amine kits were from GIBCO BRL Life Technologies (Gaithersburg, MD), tissue culture medium additives were from Biofluids (Rockville, MD), and supplies and chemicals for scintillation spectrophotometry were from Packard Instruments (Meriden, CT). Wortmannin and LY-294002 were obtained from BioMol (Plymouth Meeting, PA).

**Burn model.** Male Sprague-Dawley rats (150–200 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in the Massachusetts General Hospital animal facility under controlled conditions of 12:12 h light-dark cycles and ad libitum access to chow and water. All experimental procedures were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and the Committee on Research Animal Care, according to the guidelines in the Guide for the Care and Use of Laboratory Animals. Briefly, rats were anesthetized intraperitoneally (75 mg/kg ketamine and 1 mg/kg xylazine; Henry Schein, Port Washington, NY). The animals’ backs were shaved, and full-thickness scald burns (approximately 20% of total body surface area [TBSA]) were administered by dorsal immersion in 95°C water for 10 s. The full-thickness nature of the burn renders the injury anesthetic (9, 38). The major and minor axes of the resulting elliptical thermally injured areas were measured and used to calculate the size of the injury in square centimeters (0.7854 \( \times \) major diameter \( \times \) minor diameter). Burn size in percent TBSA was calculated via the empirical formula for total animal surface area: 11 \( \times \) weight (grams)\(^{0.631} \) (4). Control (sham) animals were immersed in 0.7854 \( \times \) body weight \( \times \) 1.5 ml/min and the liver was digested with collagenase (0.5 ml/100 g body weight) for 2 min. Between each digest, the supernatant was centrifuged at 1,500 \( \times \) g for 10 min, and the crude membrane pellet was resuspended in 10 ml SBE, filtered over a 50-mesh metal tissue sieve, and brought to a volume of 24.7 ml with SBE. Percoll (3.3 ml) was added to the suspension, thoroughly mixed, and centrifuged at 34,000 \( \times \) g for 30 min. Plasma membrane bands were harvested as described previously, diluted 1:6 (vol/vol) with SMB (250 mM sucrose, 1 mM MgCl\(_2\), and 10 mM HEPES, pH 7.5) with a Dounce homogenizer to 10 mg protein/30 min. All transport values reported are the average of the second centrifugation. Plasma membrane vesicle pellets were resuspended in SMB, and aliquots were stored at \(-80°C\) until used.

**Amino acid transport measurement in primary rat hepatocytes.** Primary rat hepatocyte amino acid transport was carried out via the duster-tray method (12) as reported previously (11). After an initial two rinses with warm Na\(^{+}\)-free Krebs-Ringer phosphate buffer (choline KRP), all transport measurements were carried out at 37°C in the presence of 50 mM L-glutamine. Amino acid transport was initiated by exposing the cells to L-[\(^{3}H\)]glutamine in either choline KRP or Na\(^{+}\)-containing KRP. Transport was terminated after 30 s by three rapid washes with 2 ml/well of ice-cold wash buffer [in mM: 119 NaCl, 25 Na\(_2\)HPO\(_4\) (pH 7.5), 3.7 mg/ml bovine serum albumin, 2.2 \( \mu \)g/ml insulin, 5 \( \mu \)g/ml transferrin, 5 mg/ml selenium, 500 mM phosphorylethanolamine/ethanolamine, 1 \( \mu \)M dexamethasone, 10 mM glucagon, trace elements (manganese, silicate, molybdenum, vanadium, nickel, and tin salts), 100 \( \mu \)M penicillin, and 100 \( \mu \)g/ml streptomycin]. The hepatocytes were quantified and assessed for viability (typically >85%) by trypan blue exclusion on a hemocytometer and diluted in RPCD to a density of 5.4 \( \times \) 10\(^4\) cells/ml. The cells (0.5 ml/well) were placed in 24-well culture plates (Costar, Cambridge, MA) previously coated with type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA) and allowed to attach for 2 h in a humidified atmosphere of 5% CO\(_2\)-95% air at 37°C. In some experiments designed to test the role of amino acids in system N regulation, hepatocytes were incubated in amino acid-free RPCD (AARPCD) made with Selectamine Kits (GIBCO BRL Life Technologies), which permitted the formulation of RPCD with all its normal constituents except amino acids.

**HPMV.** HPMV were prepared from isolated hepatocytes by a modification of the method previously described (27). The final hepatocyte pellet was homogenized in an equal volume of SEB (250 mM sucrose, 1 mM EGTA, and 10 mM HEPES, pH 7.5) with a Dounce homogenizer by 10 strokes with a loose-fitting pestle followed by multiple (200–250) strokes with a tighter-fitting pestle until >95% of the cells were visibly disrupted. The homogenate was brought to 50 ml with SEB and centrifuged at 150 g for 2 min to remove gross particulate matter and unbroken cells. The resulting supernatant was centrifuged at 1,500 g for 10 min, and the crude membrane pellet was resuspended in 10 ml SEB, filtered over a 50-mesh metal tissue sieve, and brought to a volume of 24.7 ml with SEB. Percoll (3.3 ml) was added to the suspension, thoroughly mixed, and centrifuged at 34,000 g for 30 min. Plasma membrane bands were harvested as described previously, diluted 1:6 (vol/vol) with SMB (250 mM sucrose, 1 mM MgCl\(_2\), and 10 mM HEPES, pH 7.5), and washed free of Percoll via a second centrifugation. Plasma membrane vesicle pellets were resuspended in SMB, and aliquots were stored at \(-80°C\) until used.

**Glutamine transport.** Glutamine transport was calculated from the counts per minute (cpm) per sample and the specific activity of the uptake mix (in cpm/nmol) and normalized to cellular protein content in a Microsoft Excel spreadsheet program. Transport values obtained in the absence of extracellular Na\(^{+}\) were subtracted from those in the presence of Na\(^{+}\) to yield Na\(^{+}\)-dependent rates (reported in units of nmol·mg\(^{-1}\)·protein\(^{-1}·30\) s\(^{-1}\)). All transport values reported are the average \( \pm \) SD of at least four separate determinations.

**Plasma membrane vesicle amino acid transport assay.** Initial-rate amino acid transport in HPMV was evaluated by a rapid mixing-filtering technique described previously (27) in the absence or presence of Na\(^{+}\). Uptake was initiated by...
mixing 20 µl of plasma membrane vesicles with 20 µl of Na\(^+\)- 
or K\(^+\)-containing transport buffer containing amino acid tracer in 1.5-ml centrifuge tubes using an electronic timer-
vortexer apparatus. Final concentrations in the reaction mixture were 50 mM NaCl or KCl, 1 mM MgCl\(_2\), 10 mM HEPES (pH 7.5), 50 µM L-glutamine, and 5 µCi/ml L-[\(^3\)H]glutamine. After 10 s, amino acid uptake was terminated by addition of 1 ml ice-cold wash buffer followed by immediate low-pressure vacuum filtration of the mixture over a 0.45-µm 
nitrocellulose filter to separate intravesicular from extravesicular radiolabeled amino acid. The filter was rapidly washed twice with 2 ml of ice-cold wash buffer and subjected to extraction in the same manner as that used for the hepatocytes noted above. Na\(^+\)-dependent transport rates were calculated similar to that for hepatocytes, are expressed as nanomoles of L-glutamine per milligram of protein per 10 s, and are the average \(\pm\) SD of at least four separate determinations.

Glucagon and insulin measurements. The levels of glucagon and insulin were measured in plasma from portal vein 
blood in burned and sham-burned animals after 24, 48 and 72 h, using RIA kits (Linco Research, St. Charles, MO) according to the manufacturer’s instructions.

Statistical analysis. Differences in specific measured parameters between experimental conditions were evaluated for statistical significance by a paired two-tailed t-test (Microsoft Excel) or by ANOVA with post hoc Fisher, Scheffé, and Dunnett tests (StatView Student, Abacus Concepts, Berkeley, CA) where multiple comparisons were performed. Relative differences were considered significant at \(P < 0.050\).

RESULTS

Effect of burn injury on hepatocyte glutamine transport rates. Glutamine transport rates were measured in 
hepatocytes isolated from animals at specific times after thermal or sham injury. Preliminary transport
measurements were conducted in the presence and absence of unlabeled 5 mM \(\alpha\)-(methylamino)suberic acid (MeAIB; system A-specific substrate) to assess potential contributions of system A to glutamine uptake, which can occur under certain conditions (14). However, MeAIB failed to affect Na\(^+\)-dependent glutamine uptake rates in both control and burn-influenced 
hepatocyte preparations, suggesting that all measured values were attributable to system N activity (data not shown). This is consistent with the lack of system A involvement in HPMV glutamine uptake reported in our previous study (27) using the small burn model employed here. As shown in Fig. 1, at 24 h after burn 
hepatic glutamine transport was slightly enhanced but not statistically significant compared with control hepato-
cytes (0.335 \(\pm\) 0.038 vs. 0.400 \(\pm\) 0.038 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in sham vs. burn, \(P > 0.050\)). This small difference in system N velocity in hepatocytes is consistent with the modest increase previously re-
ported in HPMV after 20% TBSA burn at 24 h (27).

After 48 h, however, hepatocyte system N activity was markedly enhanced after burn injury, where a nearly threefold increase was noted (0.267 \(\pm\) 0.043 vs. 0.783 \(\pm\) 0.076 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in sham vs. burn, \(P < 0.050\)). This burn-enhanced system N activity persisted after 72 h (0.308 \(\pm\) 0.030 vs. 0.888 \(\pm\) 0.097 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in sham vs. burn, \(P < 0.050\)).

Recognizing that burn injury is associated with a prolonged period of hypermetabolism, we sought to 
establish whether hepatic glutamine uptake remained elevated in the late postburn period. On evaluation at both 9 and 18 days after burn, it was observed that the hepatic response was indeed protracted and sustained. Glutamine transport rates in burn-influenced hepatocytes remained greater than threefold enhanced com-
pared with rates in hepatocytes from sham-burned animals after 9 days (0.345 \(\pm\) 0.045 vs. 1.102 \(\pm\) 0.124 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in sham vs. burn, \(P < 0.050\)). However, by 18 days after burn, system N activity in 
burn-influenced hepatocytes was only 36% greater than the corresponding activity in control hepatocytes 
(0.449 \(\pm\) 0.045 vs. 0.613 \(\pm\) 0.068 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in sham vs. burn, \(P < 0.050\)). At this point, the thermally injured area on the animals was appreciably healed, suggesting that this time frame may approxi-
mate the convalescence phase.

Mechanism of burn-dependent system N activation. We previously reported (27) that burn injury (20 \(\pm\) 1% 
TBSA) elicited an increase in Na\(^+\)-dependent glutamine transport activity of 35% after 24 h and 29% after 
72 h compared with activities in HPMV from sham-burned animals; however, HPMV system N activity 48 
h after burn had not been investigated. Given our current report of markedly stimulated hepatocyte gluta-
mine uptake beginning 48 h after burn, we initially wanted to determine whether this could be attributable to 
increased system N activity in the plasma membrane per se. Therefore, glutamine transport activity in HPMV 
isolated from hepatocyte preparations 48 h after sham or burn injury was determined. As shown in the inset to 
Fig. 1, burn injury elicited an increase in plasma membrane glutamine transport activity of 25% after

\[\text{Fig. 1. Na}^+\text{-dependent glutamine (GLN) transport rates in hepato-
ocytes isolated from animals at specific times after burn injury.}
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![Figure 1](http://journals.physiology.org/doi/10.1152/ajpgi.00512.2016)
Amino acid activation of system N in hepatocytes from sham-burned and scald-injured animals. Hepatocytes were isolated from sham-burned and scald-injured rats at times listed after burn injury and placed in culture for 2–3 h in RPCD medium before initial glutamine transport assay described in MATERIALS AND METHODS. Afterwards, medium was changed to amino acid-free RPCD (AAF) for 60 min, followed by a second measurement. Finally, medium was replenished with amino acid-containing RPCD (AAR) and glutamine transport was measured after an additional 60-min incubation. Na⁺-dependent rates shown are average ± SD of 4 separate determinations in at least 2 hepatocyte preparations (n = 8). *P < 0.050 vs. rates in hepatocytes from sham-burned animals.

48 h (0.032 ± 0.003 vs. 0.040 ± 0.004 nmol·mg protein⁻¹·30 s⁻¹ in sham vs. burn, P < 0.050). However, in the context of the threefold increase in hepatocyte system N activity elicited 48 h after thermal injury, the data indicated that a mechanism involving activation of existing transporters must account for the marked stimulation of glutamine uptake.

Hepatic system N activity has been shown previously to be subject to a rapid inhibitory and stimulatory modulation on removal and repletion of amino acids, respectively, from the tissue culture medium (39). This form of regulation, which is selective for system N in hepatocytes, was subsequently shown to be attributable to amino acid transport-induced increases in the cellular hydration state (7). To determine the effects of amino acids on system N activity, hepatocytes isolated from sham-treated or thermally injured animals were subjected to one or more of the following culture conditions: 1) a 2-h plating period in RPCD, 2) a subsequent 1-h incubation in AAF RPCD, and 3) a final repletion with RPCD for 1 h. System N activity was measured after each of the treatments, which were designed to test direct comparisons of glutamine transport rates, the extent to which amino acid activation contributes to the observed activities, and the ability of amino acids to stimulate the basal activity of system N, respectively. As shown in Fig. 2, at 24 h after burn, there was a slight but statistically insignificant difference between system N activity in hepatocytes isolated from the two groups in both the presence (0.335 ± 0.038 vs. 0.400 ± 0.038 nmol·mg protein⁻¹·30 s⁻¹ in sham vs. burn, P > 0.050) and absence (0.258 ± 0.018 vs. 0.268 ± 0.020 nmol·mg protein⁻¹·30 s⁻¹ in sham vs. burn, P > 0.050) of amino acids. At 48 h after burn, however, system N rates in hepatocytes from burned rats were enhanced threefold over those observed in hepatocytes from sham-burned animals (0.208 ± 0.020 vs. 0.721 ± 0.072 nmol·mg protein⁻¹·30 s⁻¹ in sham vs. burn, P < 0.050). On removal of amino acids, the disparity in system N activities vanished (0.141 ± 0.019 vs. 0.155 ± 0.009 nmol·mg protein⁻¹·30 s⁻¹ in sham vs. burn, P > 0.050), but subsequent repletion of amino acids restored the marked difference in glutamine transport rates (0.198 ± 0.015 vs. 0.533 ± 0.093 nmol·mg protein⁻¹·30 s⁻¹ in sham vs. burn, P < 0.050). Nearly identical results were obtained in burn-influenced hepatocytes isolated after 72 h, in which amino acids stimulated transport rates greater than twofold (Fig. 2).

Because postburn hypermetabolism may persist for ≥2 wk, depending on the severity of the injury (36), and given the sustained accelerated transport rates reported in Fig. 1, we further tested the extent to which this regulatory pathway exerted its influences on glutamine uptake during the chronic (9 day) and late (18 day) hypermetabolic phases. Indeed, as shown in Fig. 3, the amino acid activation pathway continued to significantly amplify system N activity after 9 days, with 60% of the total transport rates being amino acid dependent in burn-influenced hepatocytes (1.102 ± 0.124 vs. 0.440 ± 0.047 nmol·mg protein⁻¹·30 s⁻¹ in presence and absence of amino acids, respectively, P < 0.050) compared with 19% (0.449 ± 0.045 vs. 0.365 ± 0.032 nmol·mg protein⁻¹·30 s⁻¹ in presence and absence of amino acids, respectively, P < 0.050) in the corresponding controls. At 18 days after burn, 55% of total system N activity (0.613 ± 0.068 vs. 0.278 ± 0.039 nmol·mg protein⁻¹·30 s⁻¹ in presence and absence of amino acids, respectively, P < 0.050) remained amino acid-dependent rates shown are average ± SD of 4 separate determinations in at least 2 hepatocyte preparations (n = 8). *P < 0.050 vs. rates in hepatocytes from sham-burned animals. 

Sham 9 Days 18 Days

![Graph showing glutamine transport velocity](graph.png)

Fig. 3. Suppression of amino acid-dependent system N activation by hyperosmotic culture medium. Hepatocytes were isolated from sham-burned or thermally injured animals after 9 or 18 days and placed in culture for 2 h before initial glutamine transport rate measurements (RPCD). Amino acid activation was assessed exactly as described in Fig. 2, except that on amino acid repletion with RPCD, hyposmotic medium (made by addition of 300 mM sucrose) was included to suppress amino acid transport-induced cell swelling (AAR-hyperosmotic). Na⁺-dependent rates shown are average ± SD of 4 separate determinations in at least 2 hepatocyte preparations (n = 8). Sham-burned values depicted are from animals 9 days after thermal injury procedure but are not statistically different from those at 18 days after burn. *P < 0.050 vs. rates in hepatocytes from sham-burned animals; ¶*P < 0.050 vs. RPCD; ¶¶P < 0.050 vs. AAR.
dependent in burn-influenced hepatocytes compared with 25% in controls. Thus the sustained acceleration of glutamine uptake first observable 48 h after burn injury is entirely attributable to this posttranslational stimulatory pathway.

Amino acid activation of system N is known to be linked to transport-induced hepatocyte swelling and the subsequent compensatory ionic movements coupled to the regulatory volume decrease (7, 15, 40). Therefore, we further sought to determine whether the same mechanism operated in burn-influenced hepatocytes. The role of amino acid-induced cell swelling in the activation mechanism was addressed by including 300 mM sucrose (hyperosmotic media) during amino acid repletion with RPCD to suppress the transport-induced cell volume increase (7). After the 1-h incubation in AAFRPCD, hepatocytes were incubated for an additional 1 h with RPCD or RPCD plus 300 mM sucrose. As shown in Fig. 3, hyperosmotic RPCD failed to restimulate system N activity in both control hepatocytes (0.449 ± 0.045 vs. 0.231 ± 0.016 nmol·mg protein−1·30 s−1 for RPCD vs. hyperosmotic RPCD, P < 0.050) and burn-influenced hepatocytes (0.901 ± 0.090 vs. 0.392 ± 0.029 and 0.518 ± 0.067 vs. 0.299 ± 0.032 nmol·mg protein−1·30 s−1 for RPCD vs. hyperosmotic RPCD in hepatocytes isolated on days 9 and 18 after burn, respectively, P < 0.050 for all). These results indicate that, similar to normal hepatocytes (7), suppression of amino acid-induced cell swelling blocks the activation of system N in burn-influenced hepatocytes.

Signal transduction pathways of amino acid-dependent system N activation. Changes in cellular hydration not only influence system N activity but also elicit global changes in hepatic amino acid, carbohydrate, and fatty acid metabolism (17). Although this relationship has been established for nearly ten years, the signal transduction mechanisms that underlie these effects remain poorly defined. Recently, Krause and colleagues (26) reported that wortmannin, a phosphatidylinositol-3-kinase (PI3K) inhibitor, attenuated the activation of hepatic glycogen synthase and acetyl-CoA carboxylase by cell swelling. To test whether a similar link existed between amino acid-dependent system N activation and PI3K, hepatocytes isolated 48 h after thermal injury were subjected to the amino acid activation assay in the absence or presence of either of two PI3K inhibitors, wortmannin (1) or LY-294002 (37). Cells previously maintained in AAFRPCD for 30 min were subjected to an additional 30-min pretreatment with or without 0.3 or 3.0 µM wortmannin or 50 µM LY-294002, followed by amino acid repletion with RPCD in the absence or presence of either of these PI3K inhibitors. Glutamine uptake rates were measured at 5 and 60 min thereafter. Pretreatment of both control and burn-influenced hepatocytes with wortmannin decreased basal system N rates by ~20% [0.116 ± 0.015 vs. 0.149 ± 0.020 nmol·mg protein−1·30 s−1 (P > 0.050) in control hepatocytes and 0.310 ± 0.021 vs. 0.386 ± 0.036 nmol·mg protein−1·30 s−1 (P < 0.050) in burn-influenced hepatocytes in presence and absence of wortmannin, respectively]. Likewise, pretreatment with LY-294002 decreased basal activity in burn-influenced hepatocytes by 30% [0.138 ± 0.014 vs. 0.196 ± 0.017 nmol·mg protein−1·30 s−1 (P < 0.050) in presence and absence of LY-294002, respectively]. As shown in Fig. 4, on repletion with amino acids (RPCD), both 0.3 and 3.0 µM wortmannin completely abolished the rapid 80% amino acid-dependent stimulation of system N in burn-influenced hepatocytes after 5 min [0.323 ± 0.028, 0.573 ± 0.057, and 0.279 ± 0.035 nmol·mg protein−1·30 s−1 in AAFRPCD, RPCD, and RPCD + 3.0 µM wortmannin, respectively; P > 0.050 between AAFRPCD and RPCD + wortmannin], as did 50 µM LY-294002 (0.241 ± 0.010, 0.490 ± 0.065, and 0.215 ± 0.012 nmol·mg protein−1·30 s−1 in AAFRPCD, RPCD, and RPCD + 50 µM LY-294002, respectively; P > 0.050 between AAFRPCD and RPCD + LY-294002). After 60 min, 0.3 µM wortmannin inhibited the induction by 53% [0.496 ± 0.018 vs. 0.686 ± 0.051 nmol·mg protein−1·30 s−1 in presence and absence of wortmannin, respectively; P < 0.050], and 3.0 µM wortmannin by 44% [0.708 ± 0.074 vs. 0.947 ± 0.100 nmol·mg protein−1·30 s−1 in presence and absence of wortmannin, respectively; P < 0.050]. The effects of the more specific...
inhibitor LY-294002 also waned to a 43% suppression after 60 min (0.397 ± 0.024 vs. 0.524 ± 0.021 from 0.228 ± 0.025 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in presence and absence of LY-294002, respectively, \(P < 0.050\)). Collectively, the data suggest that the suppression of rapid system N activation by these two separate PI3K inhibitors is acutely effective but wanes with time.

To better understand the temporal effects of PI3K inhibition on the amino acid activation of glutamine uptake, system N activity was monitored over 30 min in the absence or presence of wortmannin or LY-294002 on repletion with amino acids in both control and burn-influenced hepatocytes. The time courses, displayed in Fig. 5, reveal that wortmannin completely abolishes the rapid twofold amino acid-dependent activation for the first 20 min in burn-influenced hepatocytes but after 30 min inhibits the response by only 50%. A similar temporal profile was obtained with LY-294002 (data not shown). In contrast, in hepatocytes from sham-burned animals the glutamine transport rates after amino acid repletion never differed by >20% at any time in the absence and presence of wortmannin. When the 20% depression of basal system N activity by wortmannin pretreatment is taken into account at time zero, the data suggest that this PI3K inhibitor fails to significantly affect the marginal 40% amino acid induction in hepatocytes from these normal, overnight-fasted animals.

Portal insulin and glucagon levels after burn. Finally, on the basis of the data it is clear that the unmasking of the latent system N activation pathway requires an in vivo “conditioning period” of >24 h. This is consistent with what is known about the latency of the amino acid-dependent regulatory pathway in general, where prior starvation of the donor animal for >24 h is required to maximize its function (7, 39). Although it is difficult to assess the quantitative contributions of individual hormones to this response in vivo, the evidence suggests that glucagon may play a role in eliciting this pathway, because both starvation and burn injury are known to alter the plasma insulin-to-glucagon ratios (43). To determine the effects of our model on these parameters, portal blood was obtained from burned and sham-burned animals after 24, 48, and 72 h. The levels of both pancreatic hormones were measured in the plasma by RIA, and the results are shown in Fig. 6. Portal insulin levels were decreased by ~50% in the burned animals compared with controls (1,187 ± 238, 846 ± 135, and 1,009 ± 209 pg/ml for control plasma vs. 490 ± 140, 501 ± 150, and 444 ± 180 pg/ml in burned animal plasma at 24, 48, and 72 h after burn, respectively, \(P < 0.050\)) on all 3 days. In contrast, portal glucagon levels in burned animals were elevated after 48 h (440 ± 50 pg/ml) vs. 24 h (216 ± 70 pg/ml, \(P < 0.050\)) and further increased after 72 h (572 ± 10 pg/ml, \(P < 0.050\)), compared with 48 h. The collective result of these burn-induced hormonal alterations was a progressive decrease in the insulin-to-glucagon ratio and thus an increasingly more “catabolic” profile over the first 72 h.

To assess the effects of glucagon on the amino acid-dependent system N induction, rats fed ad libitum were injected with 2 mg/kg of glucagon or saline (control) 4 h before hepatocyte isolation (Fig. 7). Fed rats were used because it is known that this form of transporter regulation is not visible in hepatocytes from animals not previously fasted for at least 24 h (B. P. Bode and M. S. Kilberg, unpublished results). The 4-h postinjection time was chosen because of its established stimulatory effect on system N activity (13). In hepatocytes isolated from saline-injected (control) animals, a 45% decrease in system N activity was noted on incubation in AAR RPCD for 60 min (to 0.104 ± 0.030 from 0.190 ± 0.020 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) initially in RPCD, \(P < 0.050\)), but repletion with RPCD failed to stimulate transport rates above basal values (0.117 ± 0.023 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\), \(P > 0.050\)). In contrast, hepatocytes isolated from glucagon-injected animals exhibited 34% higher initial transport rates (0.254 ± 0.020 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) as well as basal rates after 60 min in AAR RPCD (0.172 ± 0.030 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) compared with controls (all \(P < 0.050\) vs. saline-injected control). Moreover, system N rates in these hepatocytes could be restimulated to initial values on repletion of amino acids after 60 min (0.269 ± 0.030 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) vs. 0.228 ± 0.024 vs. 0.524 ± 0.021 from 0.228 ± 0.025 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) in presence and absence of LY-294002, respectively, \(P < 0.050\)).
Although the situation in burn-influenced hepatocytes is clearly more profound and complex, these data nonetheless illustrate that glucagon can influence the ability of hepatic system N to respond to amino acids.

**DISCUSSION**

The results presented here are significant for several reasons. First, the elucidation of the latent amino acid activation pathway for system N in burn-influenced hepatocytes resolves the paradoxical observation that only modest and transient increases in the corresponding liver plasma membrane activity occur (27) during the advent of the hypermetabolic phase (9) and the associated increase in hepatic amino acid extraction reported in vivo (41). Second, although amino acid activation of system N has been recognized for several years (7, 39), these studies provide the first report of a significant role for this pathway in regulating glutamine flux during a catabolic state. Finally, this work provides the first insights into signal transduction pathways linking amino acid-induced cell swelling to hepatic system N activation. Each of these points are discussed in the context of hepatic physiology during burn injury.

Among the various forms of trauma, severe burn injury gives rise to the most hypermetabolic state, resulting in appreciable increases in muscle proteolysis (35) and glutamine efflux (2) and a net negative nitrogen balance (8), the extent of which can be monitored by hepatic urea production (9, 10, 36). Both hepatic glucose- and ureogenesis rates are enhanced as a result of thermal trauma (9, 10), as is amino acid extraction by the hepatic bed (41). Given that hepatic system N activity constitutes a rate-limiting step in glutamine metabolism when intracellular utilization rates are enhanced (22, 29), our initial studies into the effect of burn injury on HPMV transport activity yielded interesting yet paradoxical results (27). In those studies, it was found that burn injury causes acute hepatic damage and stimulation of glutamine uptake, proportional to the size of the trauma. A larger (31% TBSA) injury elicited a 130% increase in HPMV glutamine transport activity after 24 h, 55% of which involved a system A component. After 72 h, the response waned to a 67% increase in HPMV glutamine uptake rates, 25% of which was system A mediated. This transient increase in HPMV Na\(^+\)-dependent glutamine transport activity corresponded with the rectification of the hepatic damage, which was no longer evident after 72 h. In contrast, the small burn injury (20% TBSA) model utilized in the present studies elicited modest increases of 35 and 29% in HPMV glutamine uptake rates after 24 and 72 h, respectively, with no discernable system A component (27). The 25% increase in glutamine uptake by 10.22\(\pm\)0.33. Although the situation in burn-influenced hepatocytes is clearly more profound and complex, these data nonetheless illustrate that glucagon can influence the ability of hepatic system N to respond to amino acids.

**Fig. 6.** Effect of burn injury on portal pancreatic hormone levels. At times indicated after thermal injury, portal blood was obtained from sham-treated or injured animals, and insulin (A) and glucagon (B) levels were measured by RIA as indicated in MATERIALS AND METHODS. *P < 0.050 vs. sham value; §P < 0.050 vs. 24-h burn value; ¶P < 0.050 vs. 48-h burn value.

**Fig. 7.** Effect of glucagon on amino acid-dependent system N activation in hepatocytes from fed rats. Male Sprague-Dawley rats fed ad libitum received an intraperitoneal injection of 2 mg/kg glucagon or an equal volume of saline (control), and hepatocytes were isolated 4 h later. After culture in RPCD for 2 h, hepatocyte glutamine transport rates were measured. After a subsequent 60-min incubation in AAF-RPCD (AAF), transport was again measured and medium was changed to amino acid-containing RPCD for an additional 60 min followed by a final glutamine transport assay. Na\(^+\)-dependent transport is shown to be 0.015 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\). Although the situation in burn-influenced hepatocytes is clearly more profound and complex, these data nonetheless illustrate that glucagon can influence the ability of hepatic system N to respond to amino acids.

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in HPMV isolated 48 h after burn (Fig. 1, inset) and lack of visible system A involvement corroborate the results from the previous study. However, this modest increase in plasma membrane system N activity and the early and transient nature of transporter stimulation after a larger burn were inconsistent with the well-established onset of the hypermetabolic phase 48 h after thermal injury in both humans (36) and rodents (9). Demonstration of the latent amino acid-dependent system N activation pathway in hepatocytes beginning 48 h after burn injury (Fig. 2) resolves this issue, because the effects of this form of regulation are not retained in isolated HPMV (7).

With respect to the coordinated integration of hepatic physiology and systemic nitrogen metabolism after burn injury, the amino acid-dependent activation of system N may serve several purposes. Primarily, amino acid stimulation of glutamine uptake may serve to maintain adequate cytoplasmic pools of this amino acid when intracellular utilization rates for ureagenesis and gluconeogenesis are enhanced (9, 22, 29). In rats, plasma concentrations of glutamine are increased during the hypermetabolic phase of burn injury compared with normal values, reflecting an augmented output from muscle (24), whereas in humans with major burn injury, plasma glutamine levels are often depressed (10). In either case, a compensatory mechanism for enhanced glutamine uptake may be necessary, as hepatic levels of this amino acid are decreased during the hypermetabolic phase (24), underscoring its rapid metabolism. The affinity of system N for glutamine is ~0.6 mM to 1 mM in humans and rats, respectively (6, 25), suggesting that this transporter operates at or below its Michaelis constant in vivo, and is therefore responsive to changes in circulating glutamine levels. From the data presented in Figs. 2–4, it is apparent that amino acids elicit a more profound activation of system N activity in burn-influenced hepatocytes compared with controls and therefore enhance the inherent efficiency of hepatic glutamine transport during this catabolic state. Thus, even in the face of a slight diminution of glucogenic plasma amino acids during the hypermetabolic phase (10), hepatic glutamine uptake would remain sufficiently stimulated via this regulatory pathway.

Secondly, the transporter may itself elicit metabolic changes via the effects of its activity on cellular hydration. Among hepatic amino acid transporters, system N exhibits the highest activity (5, 7). Thus changes in glutamine transport rates exert considerable effects on the transmembrane flux of ions and metabolites. Glutamine has been known for some time to regulate the hepatic metabolism of not only amino acids but carbohydrates and fatty acids as well (3). It has since been demonstrated that its transport-dependent effects on hepatocellular hydration underlie its regulatory properties (21, 26, 40). During the hypermetabolic phase, amino acid-dependent activation of system N may serve to increase cellular hydration, which is known to enhance flux through the glutaminase and the urea cycle (20, 21). In summary, we conclude that enhanced system N activity may serve as an autostimulatory mechanism for driving the hypermetabolic flux through the urea cycle in the burn-influenced hepatocyte, via both increased substrate delivery and transport-dependent increases in hepatocellular hydration status.

The results presented here also provide the first insights into the signal transduction mechanisms that link amino acid-induced cell swelling (Fig. 3) to hepatic system N activation. Although the association among transporter activity, cell volume, and compensatory K⁺ movements during the regulatory volume decrease has been established (7), the signal transduction pathways that underlie these processes have remained poorly understood. Recently, Low and colleagues (28) demonstrated that the cell volume modulation of muscle glutamine transport system N⁺ activity was mediated by a PI3K-dependent pathway. Also, studies by Krause and colleagues (26) have shown that the well-established activation of glycosgen synthase and acetyl CoA carboxylase by glutamine-induced hepatocyte swelling is PI3K dependent. We show here that the rapid amino acid-dependent system N activation in burn-influenced hepatocytes is mediated similarly, as evidenced by the complete inhibition of the response over the first 20 min by the PI3K inhibitors wortmannin and LY-294002 (Figs. 4 and 5). The direct activation of PI3K activity by amino acid-induced cell swelling should, however, be demonstrated in future studies to confirm the inhibitor-based results presented here and in other reports (26, 28). For example, it is unclear why this complete inhibition by both concentrations of wortmannin and LY-294002 wanes to ~50% after 30–60 min. This could reflect an inherent “leakiness” in xenobiotic PI3K inhibition with time (Fig. 4). Alternatively, this observation could be attributable to the induction of compensatory collateral pathways for transporter activation. In contrast to results in burn-influenced hepatocytes, there is only a marginal inhibitory effect of wortmannin on the amino acid induction in control hepatocytes (Fig. 5), when the 20% decrease in basal system N rates (in the absence of amino acids) by wortmannin pretreatment is taken into account (at time zero). Together, the results indicate that under normal conditions, PI3K partially regulates basal hepatic glutamine uptake rates, but this role is magnified and extended to amino acid stimulation beginning 48 h after burn injury. These findings also underscore the dynamic and complex regulation of this hepatic transport activity.

The role of specific systemic mediators in eliciting this latent PI3K-dependent regulatory pathway during the initial 48 h “catabolic priming period” remains unclear. However, several pieces of evidence suggest that glucagon may play a role. Both system N (13, 30) and glutaminase (18, 32) activities are accelerated by glucagon, which is known to increase in the plasma after burn injury (8, 34, 42). Our studies confirmed that the plasma insulin-to-glucagon ratios progressively decreased over the first 72 h after burn, attributable to an acute drop in plasma insulin levels accompanied by an incremental increase in glucagon (Fig. 6). The
impact of this progressively more catabolic hormonal profile on the amino acid-dependent system N activation might have been predicted, as it is known that starvation of donor animals for >24 h before hepatocyte isolation is required to fully manifest this pathway (7, 39). This observation is underscored by the relatively modest stimulation observed in hepatocytes from overnight-fasted sham-burned animals shown in Figs. 1–3 and 5. The effects of exogenous glucagon on the ability of hepatocytes to respond to amino acids are shown in Fig. 7, and they corroborate results obtained in earlier studies (13). Here, we extend those studies and show that both system N transport rates as well as the ability of this carrier to respond to amino acids are enhanced by glucagon in these otherwise anabolic animals. As with any in vivo event, however, it is difficult to draw conclusions on the quantitative contribution of glucagon alone to the latent activation of this regulatory pathway. Given the complex and dynamic hormonal changes that occur after burn and the interactions between potential mediators (46), more detailed and focused in vivo studies will be required to specifically address this issue. Nonetheless, these data collectively suggest that glucagon may play a role in unmasking the latency of this pathway after burn injury.

In summary, the studies presented here provide the first mechanistic insights into the accelerated hepatic glutamine uptake observed after burn injury. Through its effects on both substrate delivery and cellular hydration, the system N carrier may play a pivotal role in governing accelerated ureagenesis and nitrogen loss during the hypermetabolic phase of burn injury. Enhanced activation of this glutamine transporter by extracellular amino acids, driven by cell swelling and mediated largely by PI3K, appear to be the collective result of a 24- to 48-h “hepatic conditioning period” after burn injury by a complex mixture of catabolic hormones, including glucagon. More detailed studies on the relationships among acute-phase cytokines, catabolic hormones, and hepatic PI3K isozyme expression and function will be required to better understand the complex pathophysiology of burn injury as it relates to liver glutamine and nitrogen metabolism.

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Present addresses: R. Lohmann, Charité, Campus Virchow Clinic, Dept. of Surgery, Augustenburger Platz 1, 13353 Berlin, Germany; W. W. Souba, Pennsylvania State College of Medicine, Dept. of Surgery, 500 University Ave., Rm. C4612, MC-H051, Hershey, PA 17033.

Address for reprint requests and other correspondence: B. P. Bode, St. Louis Univ., Dept. of Biology, 3507 Laclede Ave., St. Louis, MO 63103-2010.

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