Hyperglycemia Induced by Glucose Infusion Causes Hepatic Oxidative Stress and Systemic Inflammation, But Not STAT3 or MAP Kinase Activation in Liver in Rats

Pei-Ra Ling, Claudia Mueller, Robert J. Smith, and Bruce R. Bistrian

The purpose of this study was to determine the effects of acute hyperglycemia induced by glucose infusion on oxidative stress, systemic inflammation, and several key signal intermediates in liver for the systemic inflammatory response in nonstressed rats. Rats received saline or glucose infusion (hyperglycemic clamp) for 3 hours. Rats without catheter insertion were included as an additional control for observing the effects of surgical stress. Levels of malondialdehyde (MDA) and total glutathione to assess oxidative stress were determined in liver and muscle. Proinflammatory cytokines including tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6, and alpha 1 acid glycoprotein (α1-AG) were determined in serum. The protein content and phosphorylation of extracellular signal-regulated kinase (ERK)1/2, p38 stress-activated protein kinase (p38), and signal transducer and activator of transcription-3 (STAT-3) were examined in the liver tissue with or without IL-6 stimulation. The results showed that acute hyperglycemia significantly increased MDA release and depleted total glutathione in liver but not in muscle. Hyperglycemia also significantly elevated the production of TNF, IL-1, and α1-AG, but not IL-6 in serum. However, hyperglycemia for 3 hours in vivo did not activate ERK1/2, p38 and STAT3 in liver, and also did not alter the response of these signal proteins to IL-6 stimulation. These data suggest that acute (3 hours) hyperglycemia causes hepatic oxidative stress and activates a low-grade systemic inflammation but does not affect key components of the IL-6 signaling pathway in liver.

PROVISION OF NUTRIENTS to critically ill patients is essential to slow or prevent the development of protein-calorie malnutrition and thereby reduce morbidity and mortality. However, nutritional therapy can be associated with treatment-related complications. Evidence suggests a greater adverse impact from total parenteral nutrition (TPN) over enteral nutrition (EN) on metabolic responses, particularly related to carbohydrate metabolism, with more hyperglycemia and possibly a greater risk for infectious complications. For much of past 2 decades, enteral nutrition has been the preferred route for nutrient administration largely based on these considerations.

Following the initial development of TPN, caloric intake frequently was greater than 40 kcal/kg/d, which is far in excess of actual energy expenditure of 20 to 25 kcal/kg/d.[1-3] When calorie intakes are greater than 35 kcal/kg/d or when TPN dextrose is provided in excess of 4 to 5 mg/kg/min, approximately 50 % of nondiabetic patients have been shown to develop hypergycemia (>220 mg/dL).[4] More recently, van den Berghe et al[5] demonstrated improved clinical outcome with very tight control of blood glucose (80 to110 mg/dL) among patients in the intensive care unit who received moderate caloric intakes (25 kcal/kg/d) was irrespective of route of feeding. These findings have prompted a re-evaluation of the role of hyperglycemia as a principal risk factor for adverse outcomes, rather than the route of feeding. In cell culture systems, high glucose concentrations increase the production of reactive oxygen species and tumor necrosis factor (TNF), through activation of the mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinase (ERK)1/2 and p38 stress-activated protein kinase (p38).[6-8] It was also found that the frequency of infection in people with diabetes is higher than in controls.[9] Thus, it is possible that hyperglycemia induced by overfeeding with TPN may produce its adverse impact on clinical outcome through oxidative stress and activation of systemic inflammation, which could be countered by reducing glucose infusion rates and/or controlling hyperglycemia.

Plasma glucose concentrations of greater than 200 mg/dL are commonly observed in critically ill patients, especially in the earliest stage of stress and particularly in patients with diabetes or those receiving TPN. We have shown that a somewhat higher level (~300 mg/dL) of blood glucose for 3 hours depresses immune function in rats.[10] Therefore, the current study employed the hyperglycemic clamp technique to examine whether acute hyperglycemia (300 to 350 mg/dL for 3 hours) induces evidence of oxidative stress and inflammation in nonstressed rats. Proinflammatory cytokines including TNF, interleukin (IL)-1, and IL-6, and one of the major acute-phase proteins, alpha 1 acid glycoprotein (α1-AG), were determined in serum to assess the levels of systemic inflammation. Malondialdehyde (MDA) level and total glutathione content were determined in liver and muscle tissues to assess oxidative stress. In addition, key components of the signaling cascade for systemic inflammation, including protein content and phosphorylation of ERK1/2, p38, and signal transducer and activator of transcription-3 (STAT-3), were examined in the liver tissue with or without IL-6 stimulation to assess the status and responsiveness of this signaling pathway for systemic inflammation.

From the Nutrition/Infection Laboratory, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; and the Division of Endocrinology, Brown Medical School, Providence, RI.

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Address reprint requests to Pei-Ra Ling, MD, Nutrition/Infection Laboratory, Room 503D, 21-27 Burlington Building, Beth Israel Deaconess Medical Center, 330 Brookline Ave, Boston, MA 02215.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (weight, 200 to 220 g; Taconic Farms, Germantown, NY) were acclimated in individual cages in a light-controlled room (12 hours on/12 hours off) at 22 to 24°C for 4 days in the animal facility of Beth Israel Deaconess Medical Center. During this period, animals were given free access to food and tap water. The laboratory diet contained 24 % protein, 6 % fat, and 4.5 % fiber with adequate minerals and vitamins (Rodent diet 8664, Harlan Teklad, Madison, WI).

After acclimation, a silicone catheter was inserted into the right jugular vein for glucose and saline administration under anesthesia using 40 mg/kg of ketamine plus 5 mg/kg of xylazine. The catheter was externalized in the midscapular region and attached to a flow-through swivel to permit free movement. The lines were filled with heparinized saline (16 U/mL) and sealed. After surgery, the rats were returned to metabolic cages for 4 days. During this period, animals were fed the laboratory diet and tap water ad libitum. To eliminate the effects of the surgical procedure, an additional group of rats without surgical intervention was used as a control group. Control animals also were placed individually in metabolic cages and kept on the laboratory diet and tap water ad libitum. There were no noticeable changes in eating pattern or activity levels and no obvious infection in any animal.

All procedures used in this study were approved by the Animal Care and Use Committee at the Beth Israel Deaconess Medical Center before the experiment.

IL-6

Recombinant mouse IL-6 containing less than 0.1 ng endotoxin per μg of cytokine was obtained from R&D System (Minneapolis, MN). Lyophilized mouse IL-6 was stored at −80°C and freshly made in saline with 0.1 % human albumin before the experiments.

Antibodies

Anti-STAT3 was purchased from Transduction Laboratories (Lexington, KY). Anti-phospo-STAT3, anti-ERK1/ERK2, and anti-p38 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-active MAPK (ERK1/ERK2) was obtained from Promega (Madison, WI) and anti-phospho-p38 MAP kinase antibody was purchased from New England BioLabs (Beverly, MA). Anti-IgG was purchased from Jackson ImmunoResearch Laboratory (Piscataway, NJ).

Experimental Design

On the day of study, blood from the tail vein was obtained for determination of a basal blood glucose concentration using a Glucometer Elite (Bayer Corp, Mishawaka, IN). Animals with surgically placed catheters were randomly divided into 2 groups: one received saline infusion for 3 hours (Surg − clamp) and another received 20% dextrose for 3 hours (Surg + clamp). Blood glucose levels were monitored every 15 minutes in both groups. In the animals without a surgical procedure (Con group), blood glucose was determined at 0, 60, 120, and 180 minutes. In the Surg + clamp group, blood glucose concentrations were raised to 350 mg/dL in most animals after the first 30 minutes of infusion of glucose with a variable-speed infusion pump (Harvard Apparatus, South Natick, MA), and were maintained at approximately 350 mg/dL for a further 150 minutes by a variable-rate of infusion of glucose based on the previous blood glucose concentration determined every 15 minutes.

At the end of study, animals from the Surg + clamp or Surg − clamp groups were further divided into 2 subgroups, receiving saline or IL-6 (20 μg/kg) by portal injection under anesthesia.[11] After 2 minutes of IL-6 or saline injection, animals were killed. Blood was collected, and liver and muscle tissues were harvested and immediately frozen in liquid nitrogen. Control animals were also placed under anesthesia and received saline by portal injection. Blood and tissue were collected. All samples were stored at −80°C until further analysis. The liver and muscle samples were used for determinations of levels of MDA and total glutathione, and for the levels of activation of selected signaling proteins as described.

Analytical Procedures

Serum insulin at the end of study was determined using a commercial radioimmunoassay kit with human insulin standard (ICN, Orangeburg, NY). Serum cytokines were determined using enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Camarillo, CA). Serum α1-AG concentration was determined using rat α1-AG plate kit (Cardiotech Services, Louisville, KY). The total glutathione content in liver and muscle tissues was measured using a colorimetric determination kit (Oxis International, Portland, OR). There are 3 steps to the reaction. First, the sample is buffered and the reducing agent, tris phosphine, is added to reduce any oxidized glutathione (GSSG) to the reduced state (GSH). The chromogen, 4-chloro-1-methy-7-trifluoromethylquinolinium methylsufate, is added forming thioethers with all thiols present in the sample. Upon addition of base to raise the pH greater than 13, a-β-elimination specific to the GSH-thioether results in the chromophoric thione. Then, the absorbance measured at 420 nm is directly proportional to the GSH concentration. The levels of MDA were also determined in the liver and muscle homogenate by colorimetric assay kit (Oxis International).

Liver tissues from the Surg − clamp and Surg + clamp groups were also used for determination of protein abundance and phosphorylation of ERK1/2, p38, and STAT3 with or without IL-6 stimulation to examine the effects of acute hyperglycemia (3 hours) on signaling responses for systemic inflammation. Briefly, liver samples (~1 g) were pulverized in a liquid nitrogen-cooled stainless steel mortar and pestle. The powdered tissue was transferred to a tube containing 6 mL of a buffer consisting of 20 mmol/L Tris pH 7.6, 120 mmol/L NaCl, 1 % NP-40, 10 % glycerol, 2 mmol/L sodium vanadate, 10 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 40 μg/mL leupeptin, and 100 mmol/L sodium fluoride. After homogenization in an ice bath for 45 seconds at maximum speed with a Polytron (Brinkman, Westbury, NY), the samples were mixed for 45 minutes at 4°C by end-over-end rotation and centrifuged at 200,000 × *g* for 1 hour. The clear supernatant was removed with care to avoid the overlying fat layer and stored in aliquots at −80°C for later analysis. The protein content was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels, electroblotted onto nitrocellulose membranes (BA 83, 0.2 μm; Schleicher & Schuell, Keene, NH), and incubated in 5 % bovine serum albumin for 2 hours at 37°C to block nonspecific binding. The membranes were further blotted individually with different antibodies. Resulting protein bands were detected by enhanced chemiluminescence (ECL) according to manufacturer recommendations (Amersham Pharmacia Biotech, Piscataway, NJ), identified by molecular weight, and quantitated on the ImageJ software program provided by the National Institutes of Health (NIH). To make possible the comparison of data from multiple immunoblots, the relative density of each band was normalized against an internal standard analyzed on each blot. The data from Surg − clamp group with saline injection were expressed as 1, and the relative changes in other groups were calculated against these controls.

Statistical Analysis

Data are presented as the mean ± SEM. Group means of serum parameters were compared by 1-way analysis of variance (ANOVA) using the SYSTAT statistical software package (SPSS Inc, Chicago, IL). Fisher's least-significant difference (LSD) test was used for the

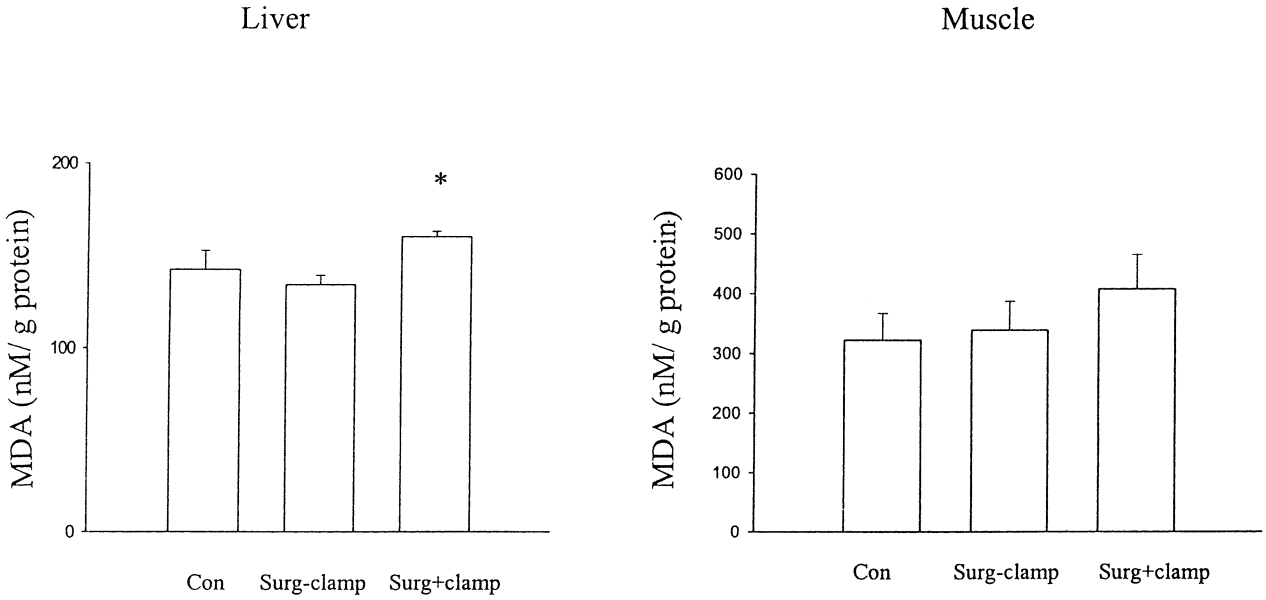

Fig 1. Effects of hyperglycemia on MDA release in liver and muscle. *$P < .05$, Surg + clamp *v* Surg − clamp by LSD. Con, animals without surgery; Surg − clamp, animals with surgery and saline infusion; Surg + clamp, animals with surgery and glucose infusion.

post-hoc test to compare the differences among groups (Con, Surg − clamp, and Surg + clamp) when ANOVA was found to be significant at the 95% confidence level. Glucose levels were compared by 1-way repeated-measures ANOVA. In addition, 2-way ANOVA (normal glucose level and hyperglycemia; saline injection and IL-6 injection) was used to compare the protein abundance and activation of ERK1/2, p38, and STAT3 in response to hyperglycemia and IL-6 stimulation in liver.

RESULTS

At the onset of the experiment, serum glucose was similar in all animals while insulin was not determined because of the limited blood sample from the tail vein. During the experiment, serum glucose concentrations were maintained at basal levels (110 ± 9 mg/dL) in both the Con and Surg − clamp groups. However, in the Surg + clamp group glucose level was significantly raised to 350 mg/dL and effectively maintained by glucose infusion. At the end of the study, serum insulin levels were significantly higher, with an approximately 3-fold increase (72 ± 9 *v* 187 ± 26 μU/mL), in the Surg + clamp group compared to the Con and Surg − clamp groups.

MDA concentration was significantly different in the liver

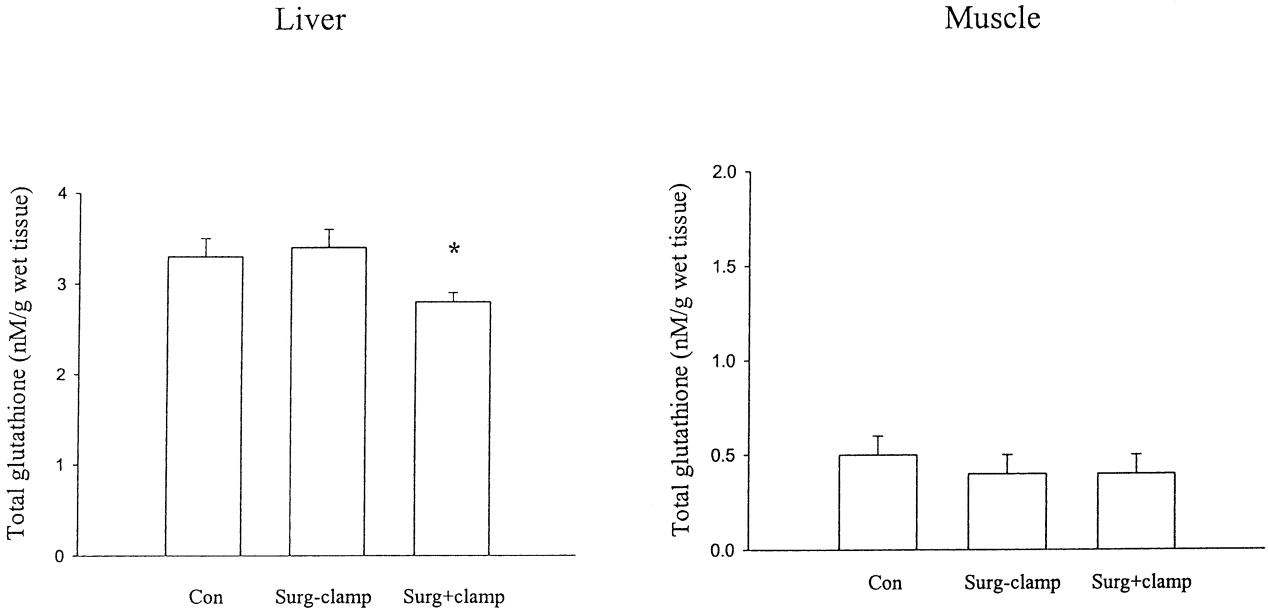

Fig 2. Effects of hyperglycemia on total glutathione content in liver and muscle. *$P < .05$, Surg + clamp *v* Surg + clamp by LSD.

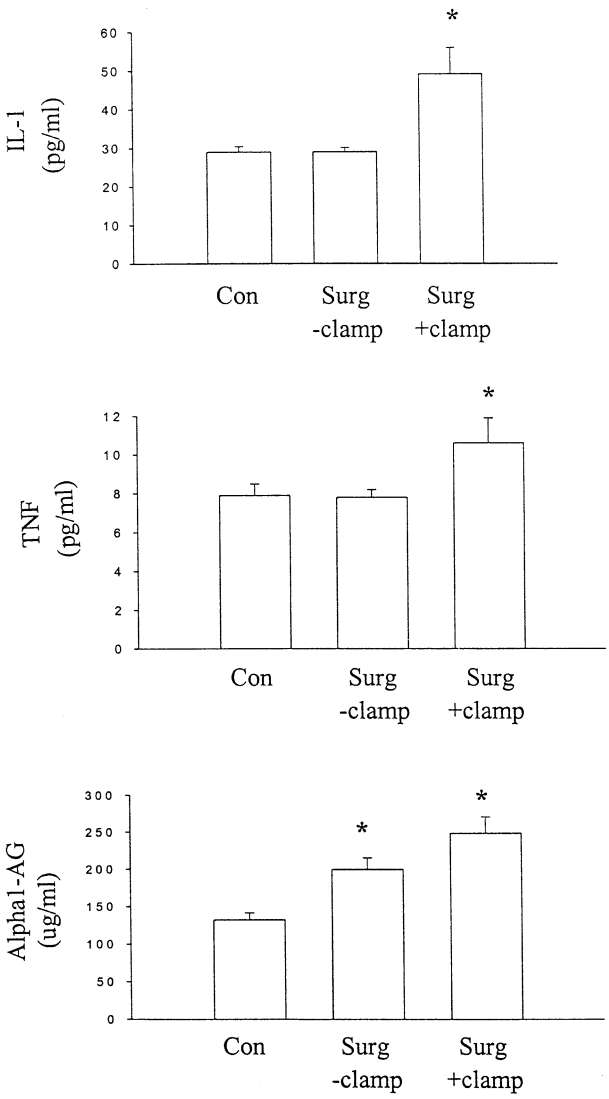

Fig 3. Effects of hyperglycemia on IL-1, TNF and α1-AG levels in serum. *$P < .05$ *v* all by 1-way ANOVA and LSD.

among the 3 groups ($P < .05$) (Fig 1). Significant increases of MDA were found in the liver in the Surg + clamp group compared to the Surg − clamp group, indicating that an increased oxidative process occurred in this tissue during the hyperglycemic condition. In muscle tissue, a slight increase of MDA was found in the Surg + clamp group, but this increase was not significantly different from Con and Surg − clamp groups. Total glutathione content in the liver was significantly decreased in the Surg + clamp group compared to Surg − clamp ($P < .05$) (Fig 2). There were no differences of total glutathione content in muscle tissue among the 3 groups.

Serum concentrations of TNF and IL-1 were not different between the Con and Surg − clamp groups, confirming that the surgical procedures and saline infusion did not alter the production of these 2 cytokines by the time of study (Fig 3). Significant increases in TNF (28 %) and IL-1 (40 %) were found in the Surg + clamp as compared to Con and Surg − clamp groups. Serum IL-6 was not detectable in any group. The level of α1-AG in serum was significantly higher in the Surg − clamp group than in Con, presumably indicating that the previous surgical procedure had initiated a systemic inflammatory response (Fig 3). However, the highest level of this protein was found in the Surg + clamp group, indicating the combined effects of hyperglycemia and earlier surgery on the production of this acute-phase reactant.

In liver tissue (Figs 4 and 5), the abundance and the levels of phosphorylation of ERK1/2, p38, and STAT3 were not different between the Surg − clamp and Surg + clamp groups with saline injection, indicating that hyperglycemia itself did not have either direct or indirect effects on these signaling proteins. In response to IL-6 stimulation, after 2 minutes of IL-6 injection, no differences in abundance of these 3 proteins (data not shown) or the levels of IL-6 activation of the 3 proteins was found in liver between the Surg − clamp and Surg + clamp groups, suggesting that normal responsiveness to IL-6 was maintained in liver after 3 hours of hyperglycemia.

DISCUSSION

In this study, the hyperglycemic clamp technique was used to achieve an elevated glucose (~350 mg/dL) level for 3 hours, and the effects of this level of blood glucose on activation of systemic inflammation and development of oxidative stress were assessed. An average 0.09 g of glucose/kg/min was infused into animals, which would provide approximately 460 calories/kg/d if continued at this rate. This amount of energy intake is about 2.5 times the required energy for rats of this size (estimated 200 kcal/kg/d for a 220-g rat from our previous experience). Therefore, the animal model used in this study not only created a hyperglycemic state in vivo, but also mimicked a common clinical episode of overfeeding principally by an acute excess glucose intake. Moreover, since an additional nonsurgical group (Con) was included in this study, the effects of hyperglycemia (Surg + clamp) on inflammation and oxidative stress by nutrition support could be distinguished from surgical stress alone (Surg − clamp).

The results show that hyperglycemia induced by short-term glucose infusion to simulate overfeeding can produce biochemical changes in the liver. The level of MDA was significantly higher in the liver with hyperglycemia. Although MDA does not specifically reflect lipid peroxidation in body fluid and tissue samples,[12] during hyperglycemia, intracellular glucose flux is increased, where it is utilized principally by anaerobic and aerobic glycolysis. The enhancement of glucose oxidation would be expected to lead the increases in the production of free radicals,[13] as this process is the principal source of these products. A high level of MDA in liver reflects an increased oxidative process occurred in this tissue under a hyperglycemic condition. The content of total glutathione in liver tissue was significantly reduced in Surg + clamp group compared to Con and Surg − clamp, also a reflection of oxidative stress. Studies have demonstrated that increased lipid peroxidation can lead to membrane dysfunction and tissue damage[14] through inactivation of receptors and membrane-bound enzymes,[15,16] and that depletion of total glutathione can increase susceptibility to chemical cytotoxicity.[17-20] Therefore, the present data suggest

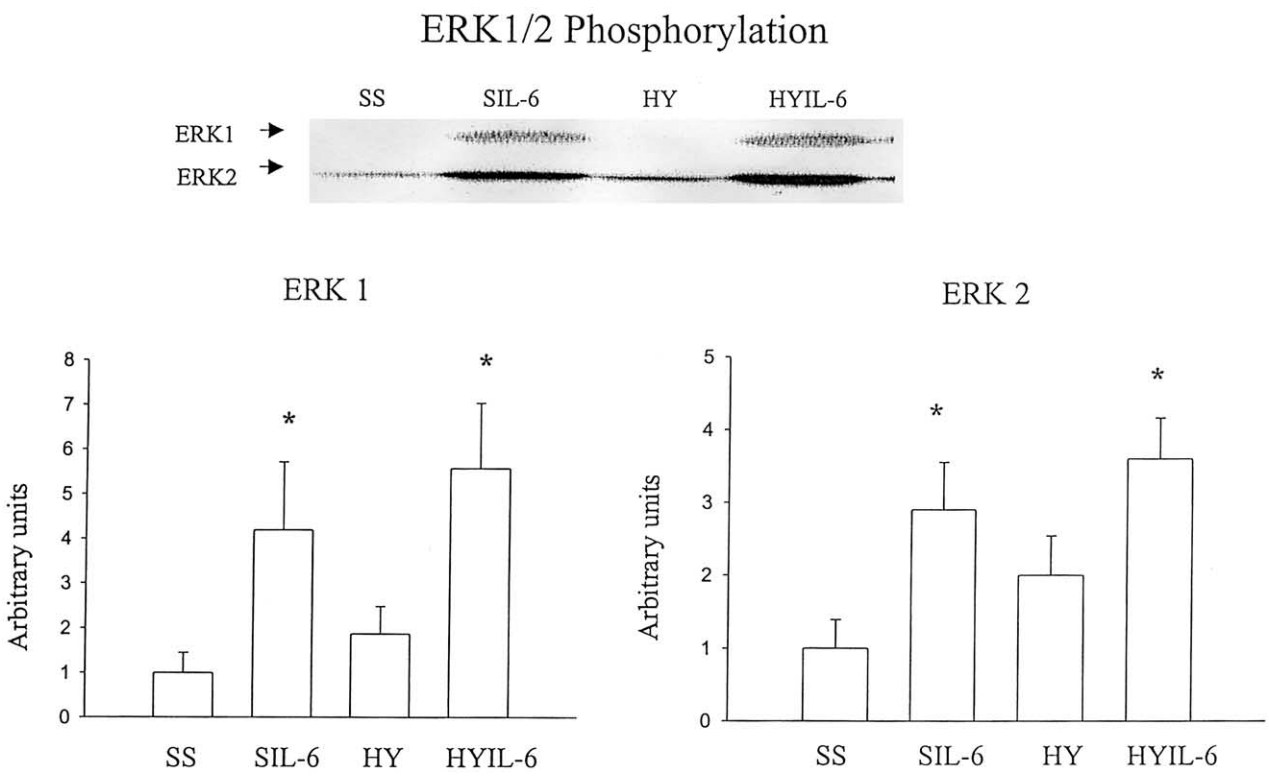

Fig 4. Effects of hyperglycemia on IL-6 activation of ERK1/2 phosphorylation in rat liver in vivo. Liver extracts were immunoblotted with phospho-specific ERK1/2 antibodies. Each lane in the blots corresponds to an individual animal in the indicated group, and quantitative analysis of multiple immunoblots in different groups is depicted in the bar graphs, where data represent the mean ± SEM for 4 rats per group expressed as relative changes in arbitrary densitometry units normalized against an internal standard. SS, animals with saline infusion and saline injection; SIL-6, animals with saline infusion with IL-6 injection; HY, animals with glucose infusion and saline injection; HYIL-6, animals with glucose infusion and IL-6 injection. *$P < .05$ *v* SS and HY by 2-way ANOVA

that acute hyperglycemia induced by glucose infusion potentially results in oxidative damage to the liver in vivo.

The lack of change in MDA production and total glutathione content in muscle tissue could reflect tissue sensitivity to hyperglycemia, because different oxidative stabilities have been observed in various cells and tissues.[21-25] It might also be due to a greater increase in glucose metabolism per gram tissue in liver over muscle as a consequence of inherent metabolic capacity and/or relative organ size.

The liver is a major site of cytokine production and the only site of acute-phase protein production. Among the many mediators of the acute-phase response, TNF and IL-1 are 2 principal proximate inflammatory cytokines, and α1-AG is one of the major acute-phase proteins in human, rat and other species. The serum concentrations of these mediators increase in response to systemic inflammation, infection and tissue injury and are correlated with increases in hepatic synthesis. In this study, serum levels of IL-1 and TNF were significantly increased in animals receiving the 3-hour glucose infusion. Simultaneously, the α1-AG concentration in serum also was significantly increased in these animals as evidence of an acute-phase response. Thus, the present results strongly suggest that short term, rapid glucose infusion, producing substantial hyperglycemia and hyperinsulinemia, induces a low-grade systemic inflammatory response. It seems most plausible that hyperglycemia increased glucose flux, enhanced glucose oxidation, and led to the oxidative stress and subsequent activation of the systemic inflammatory response evidenced by cytokine elevation and acute-phase protein production. It is also possible but less likely that cytokine activation occurred by some other mechanisms and secondarily produced oxidative stress.

Numerous studies demonstrate that acute-phase responses are individually regulated within the liver. In this study, however, the failure to detect serum IL-6 suggests that IL-6 production may be a later consequence of glucose infusion (>3 hours). Moreover, the surgical procedure led only to elevated serum α1-AG levels but not TNF and IL-1 by the time of measurement more likely reflected the effects of mild injury and the differential half-lives of these cytokines and many acute-phase proteins.

Despite these findings of hepatic oxidative stress and systemic inflammation, hyperglycemia did not activate ERKs, p38, and STAT3 in liver. This is not consistent with many in vitro studies that have demonstrated that elevated glucose levels in the surrounding media can lead to the activation of various MAP kinase cascades.[26] In rat aortic smooth muscle cells, a significant increase in p38 phosphorylation was found after 24 hours of exposure to high glucose levels.[27] A longer (72-hour) exposure of mesangial cells to high glucose was shown to significantly increase MAP kinase activity.[28,29] The activation

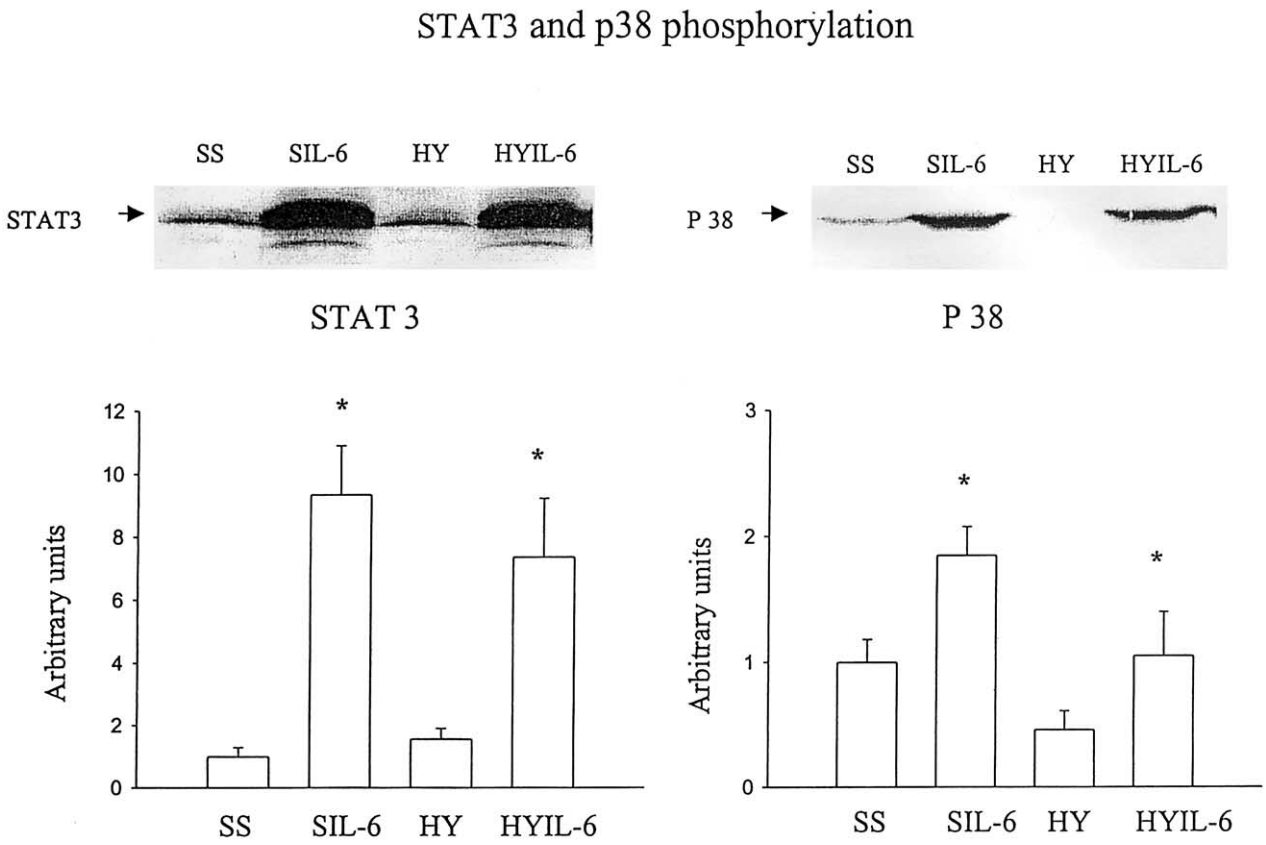

Fig 5. Effects of hyperglycemia on IL-6 activation of STAT3 and p38 in rat liver in vivo. Liver extracts were immunoblotted with phospho-specific STAT3 and p38 antibodies. Each lane in the blots corresponds to an individual animal in the indicated group, and quantitative analysis of multiple immunoblots in different groups is depicted in the bar graphs, where data represent the mean ± SEM for 4 rats per group expressed as relative changes in arbitrary densitometry units normalized against an internal standard. *$P < .05$ *v* SS and HY by 2-way ANOVA.

of STAT3 phosphorylation also was demonstrated in vascular smooth muscle cells incubated for 24 hours with high glucose levels.[30] These differences are most likely attributable to the fact that an intact animal and liver tissue were used in our study, which would provide a very different hormonal and substrate environment and differential organ participation, while various cells not including hepatocytes were used in other studies with only glucose level altered. Furthermore the fact that a short period of high glucose challenge (3 hours) was used in our study, compared to a relative longer duration (≥24 hours) in these in vitro studies may also have played a role. This concept is supported by one recent study using an ex vivo muscle incubation method showing that acute exposure of skeletal muscle for 2 to 40 minutes to high levels of extracellular glucose (25 mmol/L) did not increase phosphorylation of ERK1/2, p38, or STAT3.[31]

To examine further the effects of hyperglycemia on the signaling cascade for systemic inflammation, IL-6 was used as a stress stimulus in this study. IL-6 signaling occurs through both STAT and MAP kinase activation,[32,33] and simultaneous changes in these 2 IL-6–activated pathways could influence the different aspects (pro- or anti-inflammation) of IL-6 action.[11] The results from this study demonstrated that acute hyperglycemia did not alter protein levels of key signaling intermediates, ERK1/2, p38, and STAT3, or their activation by IL-6. Given that IL-1 and TNF also activate MAP kinases and stimulate IL-6 production in vivo, the failure to demonstrate a change in ERK1/2, p38, and STAT3 phosphorylation may further suggest little effect of hyperglycemia on the IL-6 signaling cascade itself at the time of measurement.

In summary, the present study demonstrates that acute hyperglycemia induced by intravenous overfeeding through glucose infusion can induce oxidative stress in liver tissue and produce a low-grade systemic inflammatory response in rats. These local and systemic changes have little effect on several key signal intermediates in the IL-6 signaling pathway. However, more prolonged hyperglycemia as characteristic of hypercaloric TPN could exaggerate hepatic oxidative damage and the consequent systemic inflammatory response.[22,34,35] Such changes, if they occurred, could impair the development of an appropriate systemic inflammatory response to injury, inflammation, or infection through alterations in IL-6 signaling pathway that could have adverse clinical consequences.[11] This will require further studies perhaps better modeled by longer-term employment of hypercaloric TPN.

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