Protective Effect of Glutamine from Glucocorticoid-Induced Muscle Atrophy Occurs without Alterations in Circulating Insulin-Like Growth Factor (IGF)–I and IGF-Binding Protein Levels (44157)

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Abstract. **We investigated whether the prevention of glucocorticoid-induced muscle atrophy by glutamine infusion is associated with alterations in serum levels of insulin-like growth factor (IGF)–I and its binding proteins (IGFBPs). Hormone (cortisol acetate [CA], 100 mg/kg body wt/day) and vehicle (carboxymethyl cellulose [CMC])-treated female rats were infused with either saline or glutamine (240 m*M*, 0.75 ml/hr) for a 7-day period. Glutamine infusion prevented over 70% of the skeletal muscle mass loss due to the glucocorticoid injections. Serum IGF-I concentrations, which were measured by radioimmunoassay (RIA) after acid solid-phase extraction of IGFBPs, were not significantly different among groups (range of means: 373–395 ng/ml). Saline/CA treatment resulted in a 2-fold increase in circulating levels of IGFBP-3 (38- to 50-kDa bands from ligand blotting measurements) versus the saline/CMC group. Levels of 30- to 32-kDa bands were increased by ~3-fold in the CA-treated rats. Immunoprecipitation studies suggested that the increase in the 30- to 32-kDa binding proteins were not due to elevated levels of IGFBP-1, -2, or -5. None of the treatments significantly modified circulating levels of IGFBP-4 (24 kDa). Glutamine infusion did not reverse the effects of glucocorticoids on circulating levels of 38- to 50- and 30- to 32-kDa IGFBPs. We conclude that the attenuation of glucocorticoid-induced muscle atrophy by glutamine infusion is not associated with changes in circulating levels of IGF-I or IGFBPs.**

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Glutamine availability may be one of the keys to restoring or maintaining normal or optimal muscle functioning under catabolic conditions (1). Glutamine has more functions in the body than any other amino acid. Among these, it serves as a substrate reservoir for other body tissues; it is a major fuel for immune system cells and gut mucosal cells; and its muscle concentration may function in the regulation of protein synthesis and breakdown (cf. 2). Skeletal muscle represents the primary site of glutamine synthesis. While glutamine accounts for only 5%–7% of the amino acid composition in major muscle proteins (3), glutamine comprises ~20% of the total blood amino acid pool and 50%–60% of the total muscle amino acid pool. Under a variety of conditions that produce muscle atrophy (i.e., high circulating glucocorticoids), glutamine is produced and released by muscle at high rates; glutamine concentrations may become depressed; and glutamine synthetase (GS), the enzyme that catalyzes the ATP-dependent condensation of glutamic acid and ammonia to form glutamine, is upregulated (2, 4). Recent studies in this laboratory have demonstrated that glutamine supplementation is capable of attenuating muscle wasting, the depression of myosin heavy-chain synthesis, and the glutamine synthetase in-

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duction associated with chronically elevated blood glucocorticoid concentrations (2, 4). Furthermore, there is growing evidence that a relationship may exist between glutamine and the growth hormone (GH)/insulin-like growth factor (IGF)–I axis. GH levels can be increased by an oral load of glutamine (5). GH treatment prior to trauma results in a reduction of glutamine release by muscle (6). IGF-I is also known to decrease glutamine release by skeletal muscle (7).

Among the mechanisms thought to be involved in the total body catabolism and muscle atrophy associated with glucocorticoid action is reduced IGF-I activity either directly through altered production or indirectly through increased levels of IGF-binding proteins (IGFBPs) (8–12). Unlike most peptides, IGFs circulate in association with specific IGFBPs, which are thought to modulate the biological effects of IGFs in target tissues. To date, at least six distinct IGFBPs have been purified from biological fluids and their cDNAs cloned (13). Most IGFs circulate in stable ($t_{1/2}$ = 12 hr), high–molecular weight (150-kDa), high-affinity ternary complexes together with IGFBP-3 and an acid labile subunit. Circulating levels of IGFBP-3 are increased in glucocorticoid-treated animals and humans (9, 14). Glucocorticoids also stimulate hepatic production of IGFBP-1, which is thought to be a major short-term modulator of IGF bioavailability (11). Elevated levels of IGFBPs in the circulation may reduce availability of IGFs for binding to tissue receptors, thus reducing or inhibiting their biological effects (15). Of note, IGF-I infusion is capable of preventing glucocorticoid-induced muscle atrophy (16), and glucocorticoid-induced upregulation of glutamine synthetase is attenuated by IGF-I infusion (Hickson RC, unpublished data).

Collectively, the fact that glutamine and IGF-I can both deter glucocorticoid-induced muscle atrophy and that glutamine may influence the secretion of GH and the production of IGF-I may play an important role in mediating glutamine's anticatabolic effects. In an initial line of study, we tested the hypothesis that the mechanism of muscle atrophy prevention observed with glutamine supplementation involves changes in circulating levels of IGF-I and IGFBPs.

Materials and Methods

Animal Care and Experimental Treatments. Female Sprague-Dawley rats (Sasco Animal Labs, Oregon, WI) initially weighing between 250 and 300 g were used for this study. All procedures were approved by the Animal Care Committee at UIC. Each animal was housed individually in a temperature-controlled room and provided food and water *ad libitum*. Twelve-hour light:dark cycles were maintained with lights on at 0600 and off at 1800 hr. All animals were handled daily both before and during the study in order to minimize the effect of human contact on endogenous glucocorticoid levels across treatment groups. For the experimental treatments, rats of similar body weights were arbitrarily divided into one of four groups. One group received daily injections of the dosing vehicle and constant infusion of saline *via* a catheter. A second group received glucocorticoid treatment and constant infusion of saline. The third group received the vehicle injections and glutamine infusion. A fourth group received glucocorticoid injections and glutamine infusion. For glucocorticoid treatment, subcutaneous injections of hydrocortisone 21-acetate (cortisol acetate [CA]; Sigma Chemical Co., St. Louis, MO) suspended in the vehicle (1% aqueous carboxymethylcellulose [CMC]) were administered daily at a dose of 100 mg/kg body wt/day for seven consecutive days.

For the glutamine experiments, a polyethylene sterilized catheter (PE-50) was implanted into the external jugular vein of rats in all groups at least 2 days before infusion. Animals remained moving freely during continuous infusion, by means of a 20-gauge swivel-swing infusion assembly that was connected to a 12-in. protective spring and stainless steel anchor button (Instech Laboratories, Horsham, PA). The swivel infusion assembly was secured to the animal by suturing the anchor button (which was covered with Dacron felt) to the muscles of the dorsal cervix. No harnessing jacket was used. The animals received a constant infusion of saline solution or glutamine (240 m*M*) at an infusion rate of 0.75 ml/hr for a period of seven consecutive days. At sacrifice, the collected blood was stored at –82°C until analysis. All serum analyis of IGF-I and IGFBPs were performed on the same animals.

Measurement of IGF-I, IGFBPs, and Glutamine. Serum IGFBPs were removed by treatment with 1 *M* acetic acid and C2 solid-phase extraction prior to measurement of total IGF-I by RIA, as previously reported (17). Serum IGF-I was measured by nonequilibrium double antibody immunoassay as before (18). Recombinant human IGF-I was iodinated with lactoperoxidase and repurified by immunoaffinity chromatography. Nonequilibrium double antibody immunoassay for IGF-I was performed with human IGF-I standard (BACHEM) and polyclonal antiserum against human IGF-I (NIH), as previously described (17).

IGFBPs in serum were analyzed by Western ligand blotting. Serum (2 μl) reconstituted in 50 m*M* Tris-HCL were heated in SDS/PAGE sample buffer for 10 min at 65°C prior to 13% nonreduced SDS/PAGE and electrotransfer onto 0.45-mm nitrocellulose. For ligand blotting membranes were blocked and then probed with [^{125}I]IGF-I as previously described (19). IGFBPs were identified by autoradiography and quantified by phosphorimaging (Model 445 SI, Molecular Dynamics, Sunnyvale, CA). IGFBP-3 (38–50 kDa) and nonglycosylated IGFBP-4 (24 kDa) were identified by size.

Since multiple IGFBPs have similar molecular weights of ~30 kDa on SDS/PAGE including IGFBP-1, -2, -5, and glycosylated -4, we also performed immunoprecipitation studies using specific antisera against rat IGFBP-1 (17), -4, and -5 (provided by S Shimasaki, The Whittier Institute, La Jolla, CA) (13) or bovine IGFBP-2 (Upstate Biotechnology, Lake Placid, NY) as previously described (19). Previ-

ous studies have shown that this antiserum recognizes rat IGFBP-2 and not IGFBP-1, -4, -5, or -6 in rat serum (20). In brief, washed staphylococcal protein A (Pansorbin; Calbiochem, La Jolla, CA) was incubated with antiserum, then extensively washed with Tris-buffered saline. Due to the limited availability of some antisera, test serum was pooled from each treatment group. Test serum was preincubated with fresh protein A to reduce nonspecific binding of IGFBPs, then incubated overnight at 4°C with antibody–protein A complexes. Protein A beads were rinsed, then heated in sample buffer, microfuged, and supernatant fluid loaded for 13% nonreduced SDS/PAGE. Proteins were transferred onto nitrocellulose for ligand blotting as before.

Glutamine was measured fluorometrically as cited previously (2).

Statistical Analysis. Data were analyzed by univariate analysis of variance followed by Duncan multiple range *post hoc* tests using the SYSTAT program of Wilkinson (21). Statistical significance was set at the 95% level of confidence.

Results

Effects on Body and Muscle Mass. Initial total body masses were similar in all groups (Table I). After 7 days of treatments, body masses increased by 14–15 ± 5 g ($P < 0.05$) in both groups not receiving glucocorticoids (CMC-treated groups). In CA-treated animals without glutamine, there was 2 ± 3g loss ($P < 0.05$); whereas with hormone treatment and glutamine there was a 3 ± 4–g gain in body mass.

Glucocorticoid treatment resulted in approximately 25% losses ($P < 0.05$) in several skeletal muscles (quadriceps, gastrocnemius, and plantaris) of saline-infused rats (Table I). Glutamine infusion did not enhance muscle mass in the vehicle-treated group but was able to prevent 70% ($P < 0.05$) or more of this loss in the glucocorticoid-treated group.

Serum Glutamine and IGF-I. Hormone treatment did not alter serum glutamine concentration (Table II). In both glutamine-infused groups, there was a 25% elevation ($P < 0.05$) of this amino acid.

For serum IGF-I, both vehicle-treated groups tended to have higher levels than those in the cortisol-acetate treated groups (Table II). However, these differences were modest and were not statistically significant.

Ligand Blotting of the IGFBPs. As shown in the top portion of Figure 1 and in Figure 2, glucocorticoid treatment resulted in a 2-fold increase in the 38 to 50-kDa bands (molecular weight regions are on left side of Figure 1) of the saline-infused groups. This region is representative of IGFBP-3. There also was a 3-fold induction of 30 to 32-kDa IGFBPs. These effects appear to be specific, since glucocorticoids had no effect on the 24-kDa IGFBP in saline-infused, glucocorticoid-treated animals. Glutamine had no effect on circulating IGFBPs in the vehicle-treated animals (data not shown). The bottom portions of Figure 1 and Figure 3 compare the effects of glutamine infusion versus saline infusion in CA-treated animals. No significant differences were found in the values for any band.

Since multiple IGFBPs have an apparent molecular weight of ~30 kDa on SDS/PAGE and cannot be distinguished by ligand blotting, we also performed immunoprecipitation studies using specific antibodies against IGFBP-1, -2, -4, and -5 using pooled serum from relevant treatment groups. As shown in Panel A of Figure 4, ligand blotting after precipitation of IGFBPs with specific antibodies failed to detect significant differences in circulating levels of IGFBP-2, -4, or -5 in animals given cortisol acetate with/without glutamine versus control. Precipitation of IGFBPs in pooled serum with antiserum against rat IGFBP-1 suggested that levels of IGFBP-1 may be increased in CA-treated rats and lowered down to control levels when CA-treated animals were infused with glutamine. However, as shown in Panel B of Figure 4, analysis of IGFBP-1 levels in samples collected from individual animals revealed that this apparent increase in IGFBP-1 level reflects aberrantly high levels in a single animal in the CA-treated group, and that levels of IGFBP-1 varied considerably within each group and were not significantly affected by treatment with either CA or glutamine relative to control.

Discussion

Under negative nitrogen balance conditions such as injury or starvation, where glucocorticoid production is increased, glutamine has been identified as a "conditionally"

Table I. Total Body and Skeletal Muscle Responses to Glucocortocoid and Glutamine Treatments

Variable	Vehicle-treated		Cortisol acetate–treated	
	Saline	Glutamine	Saline	Glutamine
Body mass (g)				
Before treatments	257 ± 2	256 ± 2	258 ± 1	257 ± 1
After treatments	272 ± 5	270 ± 4	231 ± 2[a]	260 ± 4
Quadriceps (mg)	1741 ± 58	1789 ± 51	1301 ± 38[a]	1703 ± 51
Gastrocnemius (mg)	1385 ± 51	1381 ± 37	1069 ± 21[a]	1302 ± 39
Plantaris (mg)	290 ± 9	284 ± 7	212 ± 5[a]	269 ± 6

Note. Values are means ± SEM. There are between seven and nine observations for each mean per group with each observation representing the data from one animal.
[a] Significantly different from cortisol acetate–treated, glutamine-infused group, $P < 0.05$.

Table II. Serum Glutamine and IGF-I Levels in Glucocorticoid-Treated and in Glutamine-Infused rats

Serum variable	Vehicle-treated		Cortisol acetate–treated	
	Saline	Glutamine	Saline	Glutamine
Gutamine (mmol/ml)	1.20 ± 0.04	1.52 ± 0.03[a]	1.22 ± 0.03	1.50 ± 0.03[a]
IGF-I (ng/ml)	398 ± 39	395 ± 21	377 ± 33	374 ± 27

Note. Values are mean ± SEM. There are at least six observations for each mean per group with each observation representing the data from one animal.
[a] Significantly different from saline-infused groups, $P < 0.05$.

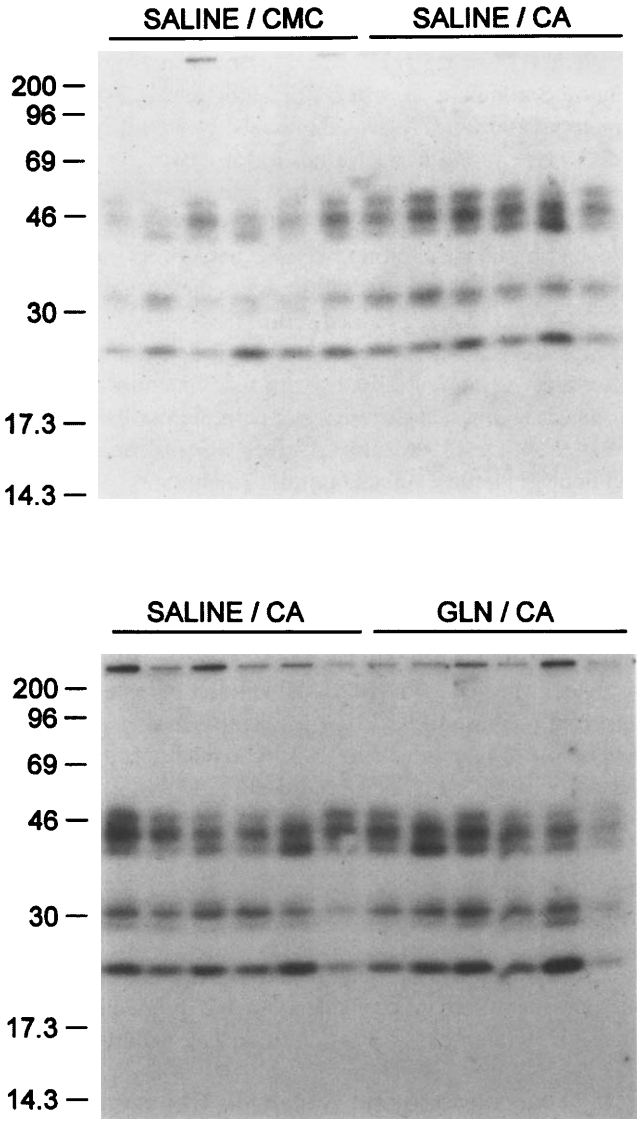

Figure 1. Ligand blotting autoradiograms of IGFBPs. Values are mean ± SEM. Values on the left side are molecular weight marker regions $\times 10^3$. (Top) Serum from saline-infused, carboxymethyl cellulose–treated (SALINE/CMC (vehicle-treated), Lanes 1–6); and saline-infused, cortisol acetate–treated animals (SALINE/CA, lanes 7–12). (Bottom) Serum from saline-infused, cortisol acetate–treated (SALINE/CA, Lanes 1–6); and glutamine-infused, cortisol acetate–treated animals (GLN/CA, Lanes 7–12). Each lane is the serum from one animal.

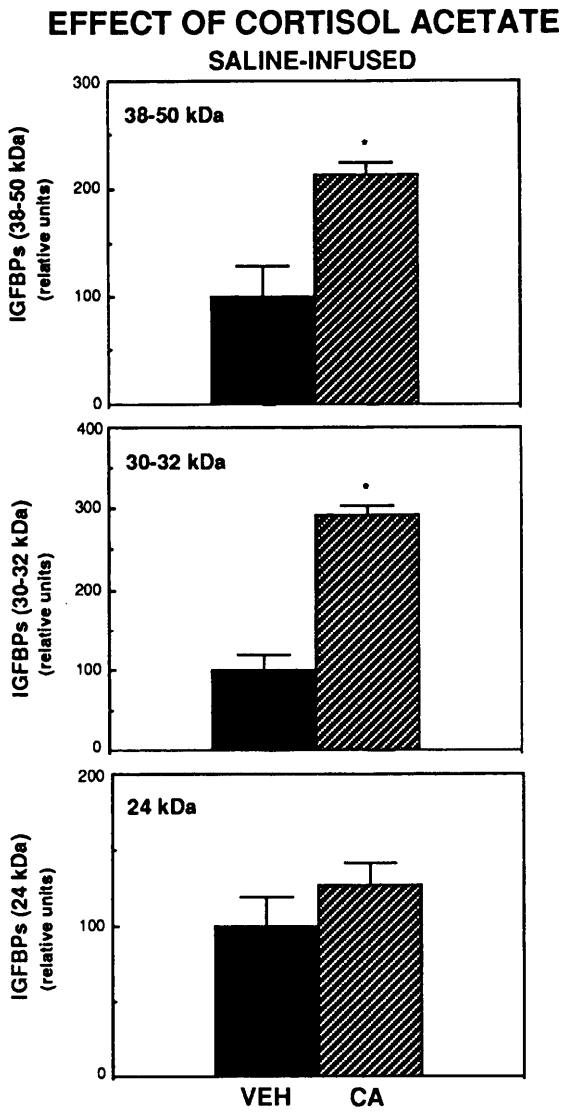

Figure 2. Phosphorimaging analysis of the effects of cortisol acetate (CA) treatment in saline (SAL)-infused rats. VEH, vehicle-treated group. There were six observations per group with each observation representing data from one animal. *Significantly different from VEH-treated, $P < 0.05$.

essential amino acid (22,23). In this state, the body's capacity to produce glutamine is exceeded by its rate of utilization. This condition subsequently leads to depleted muscle glutamine (depressed protein synthesis) and reduced circulating glutamine levels (tissue uptake exceeds production rate), which in turn contributes to muscle breakdown. Along these same lines, potential glutamine depletion exists in athletes experiencing the overtraining syndrome as well as in individuals engaged in intense interval training, where

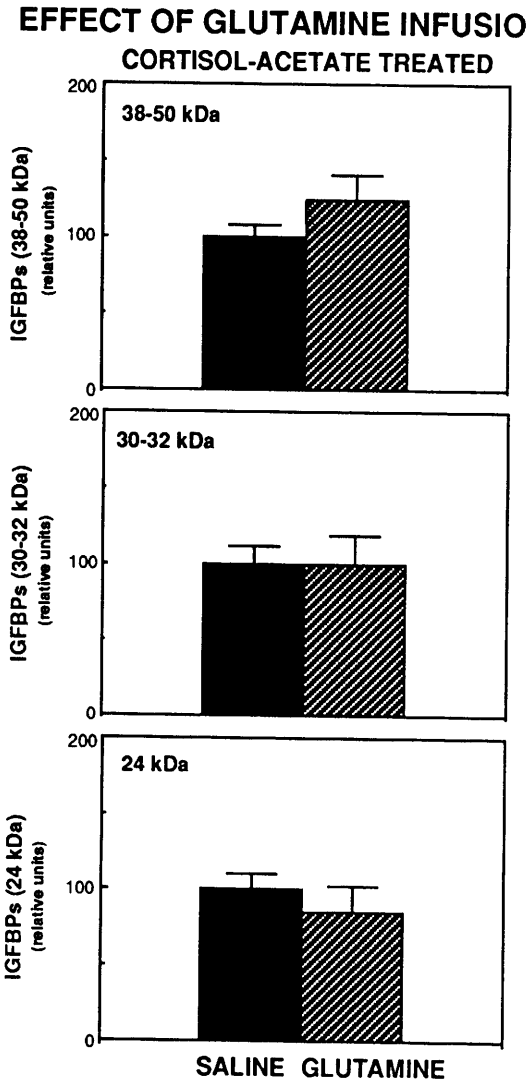

Figure 3. Phosphorimaging analysis of the effects of glutamine (GLN) infusion in CA-treated rats. There were six observations per group with each observation representing data from one animal. Abbreviations are the same as in Figure 2.

depressed blood glutamine levels have been reported (24–27). Most recently, it has been proposed that glutamine deficiency may explain the progression of muscle wasting that is found during human immunodeficiency virus infection (28). Moreover, initial support for glutamine's positive impact comes from clinical investigations. Specifically, glutamine supplementation can effectively counteract the negative nitrogen balance, the decreased concentration and size distribution of skeletal muscle ribosomes, and the muscle glutamine depletion in patients experiencing surgical trauma (1, 29, 30). The current findings further support the physiological importance of glutamine availability in deterring the total body and muscle wasting and the metabolic alterations that accompany states of glucocorticoid excess (2, 4).

Previous studies have demonstrated by ligand blotting that circulating levels of 38- to 50-kDa forms of IGFBP-3 are elevated in glucocorticoid-treated animals (9). In clinical studies, glucocorticoids also have been shown to increase the production of immunoreactive IGFBP-3 and reduce the bioavailability of IGFs in patients with tumor-related hypoglycemia (14). Glucocorticoids also are known to increase the production of IGFBP-1 in isolated hepatocytes (31) and liver-derived tumor cell lines (12). However, laboratory and clinical studies indicate that glucocorticoid treatment does not elevate circulating levels of IGFBP-1 unless insulin secretion is impaired (11, 32, 33). In the present study, we observed that circulating levels of 38- to 50 kDa forms of IGFBP-3 are increased in animals treated with CA, and that this effect of CA was not reversed by glutamine administration. Since levels of IGFs were not increased in CA-treated animals, this increase in IGFBP-3 levels may reduce the biological activity of circulating IGFs.

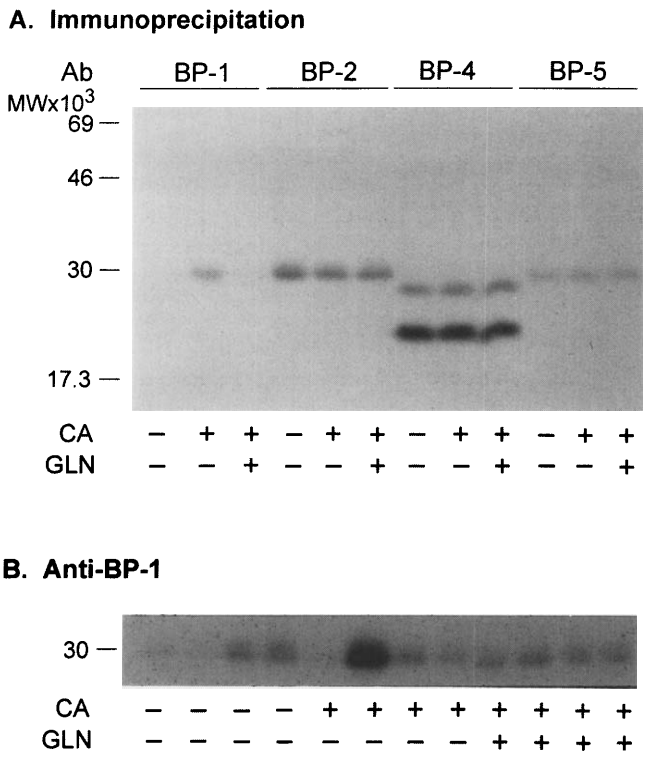

Figure 4. Immunoprecipitation of IGFBPs from serum obtained from animals treated with cortisol acetate (CA) versus carrier and infused with glutamine (GLN) versus saline. (A) IGFBPs were precipitated from pooled serum samples with specific antiserum against IGFBP-1, -2, -4, or -5, then prepared for non-reduced 13% SDS/PAGE and transfer to nitrocellulose membranes for ligand blotting with [^{125}I]IGF-I, as in Figure 1. (B) IGFBP-1 was immunoprecipitated from serum samples from individual animals treated with/without CA and/or GLN, using specific anti-rat IGFBP-1 antiserum. Precipitated proteins were prepared for SDS/PAGE and ligand blotting as before.

Ligand blotting also revealed an increase in circulating levels of 30-kDa IGFBPs in CA-treated animals. Immunoprecipitation studies demonstrated that this increase in 30-kDa IGFBPs was not due to changes in levels of IGFBP-1, -2, -4, or -5. Previous studies have demonstrated that circulating proteases can degrade IGFBP-3, and that ~30-kDa proteolytic fragments of rat IGFBP-3 are detectable by ligand blotting (34). We have previously found that the avail-

able antiserum against rat IGFBP-3 (gift from N. Ling and S. Shimasaki) only weakly precipitates IGFBP-3 proteins from rat serum (20). Thus, it remains possible that the increase in 30-kDa IGFBPs observed in CA-treated animals reflects the presence of increased amounts of proteolytic fragments of IGFBP-3. Since treatment with glutamine did not affect circulating levels of IGFBPs in CA-treated animals, it appears unlikely that glutamine administration modulates the availability of circulating IGFs through changes in the IGFBP system.

Several lines of evidence prompted the investigation of IGF-I as a source of glutamine interference of glucocorticoid action. IGF-I has been identified as a growth hormone (GH)–dependent factor, which is expressed in many tissues and is thought to mediate most of the growth-promoting effects of GH through both endocrine and autocrine/paracrine mechanisms. Treatment with IGF-I alone, or in combination with GH, helps to restore anabolism in a variety of catabolic states, including malnutrition, burns, surgery, and glucocorticoid administration (16, 35–37). IGF-I levels are regulated by local and non-GH factors, including exercise (38, 39) and protein nutrition (40). Thus, it seemed reasonable to speculate that mechanisms which increase the availability of endogenous IGFs might help to prevent catabolic complications of glucocorticoid excess, and the ability of oral glutamine to stimulate growth hormone secretion suggested that IGF-I could also be positively influenced.

However, while glutamine infusion was effective in preventing a large portion of glucocorticoid-induced muscle atrophy, we found that it was ineffective in altering IGF-I levels. Several animal studies indicate that glucocorticoids may reduce GH secretion (cf., 9, 41) and impair the ability of GH to stimulate IGF-I production in rats (8). Nevertheless, other data show that glucocorticoids may impair anabolism without lowering total circulating levels of IGF-I (11, 16). Conversely, in dose-response studies GH has been shown to stimulate growth and to increase tissue IGF-I content before changes in serum levels of IGF-I are detected (42). In this context, it is important to note that most circulating IGFs are thought to be produced by the liver (43), and that glutamine as well as glucocorticoids might well exert important effects on the production of IGFs and/or IGFBPs in extrahepatic tissues, which may not be reflected in circulating levels of IGF-I or IGFBPs (14). Additional studies are indicated to evaluate the possibility that alterations in IGF-1 or IGFBP expression at the tissue level may play an important role in mediating the anticatabolic effects of glutamine in a state of glucocorticoid excess.

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