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Glucosamine Inhibits Inducible Nitric Oxide Synthesis

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Glucosamine is widely used in Europe for treatment of arthritis in humans. Based on recent findings that excess production of nitric oxide (NO) by inducible NO synthase (iNOS) mediates the pathogenesis of arthritis, we hypothesized that glucosamine may inhibit NO synthesis. To test this hypothesis, we used an *in vivo* rat model of lipopolysaccharide (LPS)-induced inflammation. Intravenous administration of D-glucosamine (0.5 mmol/kg) 6 h before, at the time of, and 6 h after intraperitoneal LPS injection (1 mg/kg) decreased urinary excretion of nitrate by 31 and 48%, respectively, at days 1 and 2 post LPS administration. When cultured macrophages were treated with LPS (1 μg/ml) to induce iNOS expression, addition of 0.1, 0.5, 1, and 2 mM D-glucosamine decreased NO production by 18, 38, 60, and 89%, respectively. Glucosamine had no effect on cellular arginine, NADPH or tetrahydrobiopterin concentrations, but dose-dependently suppressed iNOS protein expression. Similar decreases in iNOS protein occurred in spleen, lung, and peritoneal macrophages of glucosamine-treated rats. These studies demonstrate that glucosamine is a novel inhibitor of inducible NO synthesis via inhibition of iNOS protein expression, and provide a biochemical basis for the use of glucosamine in treating chronic inflammatory diseases such as arthritis. © 2000 Academic Press

Key Words: **glucosamine; nitric oxide synthesis; arthritis.**

Arthritis, a group of chronic inflammatory diseases characterized by destruction of joint cartilage, affects more than 50 million Americans (1). However, the mechanisms responsible for this joint cartilage destruction are largely unknown. In the U.S., pain-relieving drugs, nonsteroidal anti-inflammatory drugs, or steroids are commonly used to treat arthritis (1). These medications help relieve symptoms but cannot cure arthritis, and can cause serious side effects (2). As the disease becomes progressively more severe, hips and knees are surgically replaced with nonpermanent artificial ones. Interestingly, glucosamine, a natural metabolite synthesized from glutamine and fructose-6-phosphate, has been widely used in Europe to treat osteoarthritis in humans and animals (4–8). In recent years, there has been growing interest in the use of glucosamine to treat arthritis in the U.S., and its beneficial effect has been reported for both humans and animals (9–12). The clinical efficacy of glucosamine in treating arthritis is thought to result from increased synthesis of structural proteoglycans in joint cartilage (9, 13).

Recent studies have demonstrated increased production of nitric oxide (NO) from arginine and enhanced expression of inducible NO synthase (iNOS) in humans with arthritis (14–16) and animal models of adjuvant-induced arthritis (17–21). Furthermore, inhibition of inducible NO synthesis by N^G-monomethyl-L-arginine or *N*-iminoethyl-L-lysine (inhibitors of NOS) at the time of induction of arthritis has been shown to profoundly reduce the synovial inflammation, joint swelling, and tissue damage in rats (17–19), implicating a critical role for excess NO production by iNOS in mediating the pathogenesis of arthritis. In light of these findings, we hypothesized that glucosamine is an inhibitor of inducible NO synthesis. As an initial test of this novel hypothesis, the present study was conducted using an *in vivo* rat model of LPS-induced inflammation (22, 23) and the RAW 264.7 murine macrophage cell line (24).

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL (Grand Island, NY) and fetal bovine serum from Summit (Greeley, CO). D-glucosamine · HCl, L-glutamine, LPS (from *Escherichia coli* serotype 0127:B8), D-glucose, HEPES, NADPH, tetrahydrobiopterin, EDTA, *o*-phthaldialdehyde, penicillin G, chloramphenicol, and amikacin were obtained from Sigma Chemicals (St. Louis, MO).

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Effect of glucosamine on in vivo NO synthesis in a rat model of LPS-induced inflammation. Sixteen 45-d-old male Sprague-Dawley rats were obtained from Harlen, Inc. (Houston, TX), and placed in metabolism cages in a temperature- and humidity-controlled facility on a 12-h light/12-h dark cycle. Animals had free access to double-distilled and deionized water and a nitrite- and nitrate-free semipurified diet (Research Diets, New Brunswick, NJ), as previously described (22). At 60 days of age, rats (330–350 g) were divided randomly into two groups of 8, and received intravenous administration of 0 or 0.5 mmol D-glucosamine · HCl per kg body wt via the tail vein (in 0.24 ml of 0.15 M phosphate-buffered saline, pH 7.4), 6 h before, at the time of, and 6 h after intraperitoneal injection (1 mg/kg body wt) of LPS. This dose of glucosamine (1.5 mmol/kg body wt/day) was chosen for the following reasons. First, oral administration of glucosamine (1.57 mmol/kg body wt/day) has previously been shown to be effective in preventing arthritis in rat models (8). Second, our preliminary study indicated that intravenous administration of glucosamine at the dose of 1.5 mmol/kg body wt/day reduced urinary excretion of nitrate (the major end product of NO oxidation *in vivo*) by LPS-treated rats. Twenty-four h urine collections were performed for 4 days before and after LPS administration. Urine was collected into brown bottles containing antibiotics (60 mg penicillin G, 100 mg chloramphenicol, and 6 mg amikacin) (22, 25). Urine samples were diluted 20–200 times with deionized-distilled water for nitrate analysis, as previously described (26).

Effect of glucosamine on plasma concentrations of glucosamine in a rat model of LPS-induced inflammation. Rats received intravenous administration of 0 or 0.5 mmol D-glucosamine · HCl per kg body wt, 6 h before, at the time of and 6 h after intraperitoneal LPS injection (1 mg/kg body wt), as described above. Blood (50 μl) was drawn from the tail vein into a heparinized hematocrit tube (25) at 0, 3, 12, 24, and 48 h after the initial glucosamine injection. The blood samples at 0 and 12 h were taken immediately before glucosamine administration. Plasma samples (25 μl) were deproteinized with 25 μl of 1.5 M $HClO_4$. The acidified solution was neutralized with 12.5 μl of 2 M K_2CO_3 and then centrifuged at 10,000*g* for 1 min. The supernatant was diluted 25 times with HPLC-grade water, and then used for analysis of amino acids and glucose by HPLC and enzymatic methods (22, 25), respectively. Glucosamine was analyzed by an HPLC method involving the precolumn derivatization of glucosamine with *o*-phthaldialdehyde, as described previously (27), with modifications in the solvent gradient. The HPLC mobile phase was as follows: 0 to 14 min, 86% Solvent A (0.1 M sodium acetate, 0.5% tetrahydrofuran, 9% methanol, pH 7.2) and 14% Solvent B (methanol); 14.1 to 18 min, 100% Solvent B; 18.1 to 24.5 min, 86% Solvent A and 14% Solvent B. The retention time of glucosamine was 13.6 min, as determined with an authentic glucosamine standard.

Effect of glucosamine on inducible NO synthesis in RAW 264.7 macrophages. To elucidate the mechanism for the glucosamine inhibition of inducible NO synthesis, we employed the RAW 264.7 murine macrophage cell line (American Type Culture Collection, Manassas, VA). Cells (2×10^5) were cultured for 6 h in 0.5 ml phenol red-free DMEM containing 20 mM HEPES (pH 7.5), 1 mM L-glutamine, 11 mM D-glucose, 0.4 mM L-arginine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 1% dialyzed fetal bovine serum, and 0, 0.1, 0.5, 1, or 2 mM D-glucosamine. After this 6 h period of preexposure, cells were cultured for 24 h in fresh medium containing 0, 0.1, 0.5, 1, or 2 mM D-glucosamine and 0 or 1 μg/ml LPS. At the end of the 24-h culture period, conditioned media were used for the determination of nitrite and nitrate using the Griess reagent (26) and cells were used for iNOS protein analysis and determination of arginine, NADPH, and tetrahydrobiopterin levels (see below). In all experiments, culture medium without cells was used as a blank for nitrite and nitrate analysis.

Western blot analysis of iNOS. Analysis of iNOS in cultured RAW 264.7 cells was performed essentially as described for ecNOS analysis by Hood *et al.* (28). Six micrograms of total cell protein was loaded per lane of a 7.5–16% gradient gel. The primary antibody, mouse anti-iNOS (Clone 54, BD Transduction Laboratories, San Diego, CA), was used at a 1:5000 dilution in Tris–buffered saline and the secondary antibody, peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), was used at a 1:60,000 dilution. Peroxidase activity was visualized using Super Signal West Dura Extended Duration Substrate(Pierce, Rockford, IL), according to the manufacturer's directions, with Kodak Biomax ML film (Kodak, Rochester, NY). Blots were scanned using a UMAX S6E scanner (UMAX Data Systems, Hsinchu, Taiwan) with VistaScan software (Kodak).

Peritoneal macrophages were isolated from rats 24 h post LPS administration as previously described (29). Thirty micrograms of total cell protein was loaded per lane of a 7.5–16% gradient gel. The primary antibody, polyclonal rabbit anti-iNOS (BD Transduction Labs), was used at a 1:5000 dilution and the secondary antibody, peroxidase-labeled donkey anti-rabbit IgG (Jackson), was used at a 1:50,000 dilution. Bands were visualized as described above.

To detect iNOS protein in lung and spleen, organs were removed from rats 24 h post LPS administration and frozen immediately in liquid nitrogen. Lung and spleen were chosen because our previous study indicated that these two organs exhibit the greatest iNOS activity of all major tissues of LPS-treated rats (22). A portion of frozen tissue was then solubilized in lysis buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 0.1% SDS, 1% deoxycholate, 1% NP-40, 0.14 M NaCl, 0.5 mg/ml Pefabloc, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) using a Polytran tissue homogenizer. One milligram of protein from the tissue lysate was immunoprecipitated with 4 μg of monoclonal anti-iNOS (Clone 6, BD Transduction Labs) in 20 mM Tris–HCl, pH 8.0, overnight at 4°C and immunoglobulin isolated with Protein A Sepharose CL-4B. Bound protein was eluted with electrophoresis sample buffer and loaded on a 7.5–16% gel. The primary antibody, polyclonal rabbit anti-iNOS (BD Transduction Labs), was used at a 1:5000 dilution and the secondary antibody, peroxidase-conjugated donkey anti-rabbit IgG (Jackson), was used at a 1:50,000 dilution. Bands were visualized as described above.

Western blot analysis of actin. Ten micrograms of peritoneal macrophage lysate protein, 5 μg of lung homogenate protein, and 10 μg of spleen homogenate protein were loaded per lane of a 7.5–16% gradient gel. The primary antibody, monoclonal anti-actin (Clone AC-400, Sigma) was used at a 1:500 dilution and the secondary antibody, peroxidase-conjugated donkey anti-mouse IgG (Jackson), was used at a 1:50,000 dilution. Bands were visualized as described above.

Determination of arginine, NADPH and tetrahydrobiopterin. Arginine levels in cells were determined by an HPLC method involving precolumn derivatization with *o*-phthaldialdehyde (30). NADPH levels in cells were determined by the HPLC method of Stocchi *et al.* (31) except that 1 mM bathophenanthroline-disulfonic acid (a divalent metal chelator) was used to prevent oxidation of NADPH by iron and that NADPH was detected by a fluorometer (excitation 340 nm, emission 460 nm) to improve the assay sensitivity (32). Tetrahydrobiopterin was determined by the HPLC method of Fukushima and Nixon (33), except that 5 mM dithioerythritol (an antioxidant) was included in the cell extraction step (32).

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) (34). Differences between means were determined by the Student-Newman-Keuls (SNK) multiple comparison test (34). Probability values <0.05 were taken to indicate significant differences.

RESULTS AND DISCUSSION

Effect of glucosamine on inducible NO synthesis in a rat model of LPS-induced inflammation. Glucosamine has been widely used in Europe for treatment of arthritis in humans and animals (4–8). The clinical

TABLE 1

Effect of Glucosamine on *in Vivo* Inducible Nitric Oxide Synthesis in Rats Treated with Lipopolysaccharide[1]

Rats	Time post-LPS injection (day) 1	2	3
	μmol/kg body wt/24 h		
Control	173 ± 19[a]	46.3 ± 5.1[b]	3.1 ± 0.44[c]
Glucosamine-treated	119 ± 14*[a]	24.1 ± 2.2*[b]	2.7 ± 0.35*

[1] All values are reported as mean ± SEM (n = 8) and were analyzed by two-way ANOVA. Inducible NO synthesis by LPS-treated rats was calculated on the basis of urinary nitrate excretion before and after LPS administration. Values of urinary nitrate excretion before LPS injection were 8.4 ± 0.76 and 8.1 ± 0.71 μmol/kg body wt/24 h. *$P < 0.01$, different from the control group. a–c, means with different letters within a treatment are different ($P < 0.01$).

TABLE 3

Effect of Glucosamine on Inducible Nitric Oxide Synthesis in RAW 264.7 Murine Macrophages[1]

Medium [glucosamine] (in mM)	Production of nitrate plus nitrite (nmol/24 h/10^6 cells)
0	3.29 ± 0.22[a]
0.1	2.70 ± 0.17[b]
0.5	2.04 ± 0.12[c]
1.0	1.32 ± 0.10[d]
2.0	0.35 ± 0.04[e]

[1] All values are reported as mean ± SEM (n = 6) and were analyzed by two-way ANOVA. RAW 264.7 cells were cultured for 24 h in the presence of 1 μg/ml LPS and 0 to 2 mM D-glucosamine. Conditioned media were used for nitrite and nitrate analysis. a–e, means with different letters are different ($P < 0.01$).

efficacy of glucosamine is thought to result from increased synthesis of structural proteoglycans in joint cartilage (9, 13). However, on the basis of the recent finding that excess NO production by iNOS mediates the pathogenesis of arthritis (17–19), we hypothesized that glucosamine could inhibit inducible NO synthesis. As an initial test of this novel hypothesis, we employed an established rat model of LPS-induced inflammation (22, 23). As previously reported by us and other investigators (22, 23), urinary excretion of nitrate [the major end product of NO oxidation *in vivo* (35)] was markedly increased within 24 h after LPS administration (Table 1), and this provided a useful animal model for determining whether glucosamine suppresses inducible NO synthesis *in vivo*.

Glucosamine administration increased plasma glucosamine concentration from a basal value of 1.5 μM to 286 μM at 3 h post glucosamine administration (Table 2), indicating a rapid rise in plasma glucosamine levels. Glucosamine treatment had no effect on plasma concentrations of arginine, ornithine, or glucose (data not shown), suggesting that glucosamine, at the dose used, did not affect arginine catabolism via arginase or glucose utilization *in vivo*. However, glucosamine decreased urinary nitrate excretion by 31 and 48%, respectively, at days 1 and 2 post LPS treatment (Table 1). By day 3 post LPS administration, when inducible NO synthesis was nearly absent (22, 23), there was no difference in urinary nitrate excretion between control and glucosamine-treated rats. These results indicate that glucosamine treatment inhibited inducible NO synthesis *in vivo*.

Effect of glucosamine on inducible NO synthesis by RAW 264.7 macrophages. To firmly establish a role for glucosamine in inhibiting inducible NO synthesis and to begin to elucidate the mechanism involved, we employed the RAW 264.7 murine macrophage cell line, which is known to express iNOS in response to stimulation by inflammatory cytokines and bacterial endotoxin (24). There was no detectable production of nitrite or nitrate by RAW cells in the absence of LPS. However, in response to stimulation by LPS, RAW cells produced large amounts of NO (Table 3). Culturing RAW cells in the presence of 0.1, 0.5, 1, and 2 mM glucosamine resulted in a dose-dependent decrease in inducible NO synthesis by 18, 38, 60, and 89%, respectively, compared with the absence of glucosamine (Table 3). This data, taken together with the *in vivo* find-

TABLE 2

Plasma Concentrations of Glucosamine in Rats Treated With or Without Glucosamine[1]

Rats	Time post initial glucosamine administration (h) 0	3	12	24	48
			Glucosamine (μM)		
Control	1.36 ± 0.15	1.43 ± 0.13	1.40 ± 0.17	1.29 ± 0.14	1.22 ± 0.18
Glucosamine	1.45 ± 0.17*[a]	286 ± 21*[d]	120 ± 5.2*[c]	8.73 ± 1.0*[b]	1.64 ± 0.25*[a]

[1] All values are reported as mean ± SEM (n = 6) and were analyzed by two-way ANOVA. Rats received intravenous administration of 0 or 0.5 mmol D-glucosamine per kg body wt, 6 h before, at the time of, and 6 h after intraperitoneal LPS injection (1 mg/kg body wt). Blood samples at 0 and 12 h were taken immediately before glucosamine administration. *$P < 0.01$, different from the control group. a–d, means with different letters within a treatment are different ($P < 0.01$).

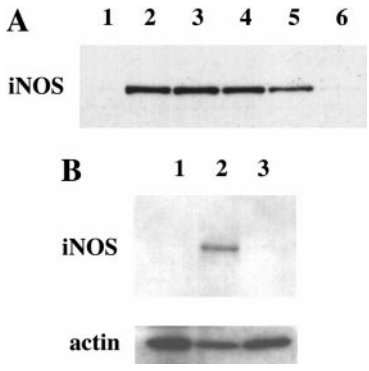

FIG. 1. Western blot analysis of iNOS in cultured and peritoneal macrophages. (A) RAW 264.7 macrophages were cultured for 24 h in the absence or presence of 1 μg/ml LPS (+LPS) and 0 to 2 mM D-glucosamine, and were then used for Western blot analysis of iNOS. Cells cultured in the absence of LPS served as a negative control for iNOS expression. Key to the lanes: (1) no LPS treatment, (2) +LPS/0 mM glucosamine, (3) +LPS/+ 0.1 mM glucosamine, (4) +LPS/+ 0.5 mM glucosamine, (5) +LPS/+1 mM glucosamine, (6) +LPS/+ 2 mM glucosamine. (B) Peritoneal macrophages were isolated from untreated rats (no LPS) and rats treated with LPS (1 mg/kg body wt) with or without glucosamine treatment (1.5 mmol/kg body wt/day). Cell lysates were used for Western blot analysis of both iNOS and actin expression.. Key to the lanes: (1) no LPS treatment, (2) LPS treatment alone, (3) LPS treatment + glucosamine treatment.

ing, indicates that glucosamine is a novel inhibitor of inducible NO synthesis.

L-arginine, NADPH and tetrahydrobiopterin are required for NO synthesis by iNOS, which is calcium independent (36). To provide a metabolic basis for the inhibition of inducible NO production by glucosamine, we determined cellular concentrations of these cofactors and this substrate of iNOS, along with iNOS protein levels. Glucosamine had no effect on cellular concentrations of arginine, NADPH or tetrahydrobiopterin (data not shown). However, Western blot analysis showed that glucosamine treatment reduced iNOS protein levels in RAW cells in a dose-dependent manner (Fig. 1A). At 2 mM glucosamine, iNOS protein was not detectable. A similar result was obtained when peritoneal macrophages from the glucosamine-treated animals were analyzed (Fig. 1B). No iNOS was detected without LPS induction or when rats were treated with glucosamine concurrently with LPS induction. The inhibition of iNOS protein expression appeared to be specific since expression of actin was not affected by glucosamine treatment (Fig. 1B). Our results suggest that glucosamine inhibits the expression of iNOS in macrophages, and this is likely to be the biochemical basis for the inhibition of inducible NO synthesis.

We have previously shown that lung and spleen exhibit very high activities of iNOS activity in response to LPS injection (22). In this study, LPS induced the expression of iNOS protein in these organs (Fig. 2). Glucosamine treatment reduced the expression of iNOS protein in both spleen and lung. Actin expression was not changed by glucosamine treatment, suggesting a specific effect of glucosamine on iNOS expression. This suppression by glucosamine of iNOS protein expression in organs as well as peritoneal macrophages would explain our finding that NO synthesis *in vivo* was markedly reduced in glucosamine-treated rats on days 1 and 2 post LPS administration (Table 1). Thus, in contrast to all known competitive and noncompetitive inhibitors of iNOS (37), glucosamine is a novel inhibitor of NO synthesis, acting presumably by inhibiting the expression of the gene for iNOS. Further studies are necessary to determine whether glucosamine inhibits iNOS transcription and/or translation.

Possible physiological and pharmacological significance. iNOS is induced by inflammatory cytokines and bacterial endotoxin (38) and is overexpressed in target cells in various immune-mediated diseases (39). While NO production by iNOS plays an important role in killing pathogenic bacteria and viruses (37), there is evidence suggesting that overproduction of NO by iNOS contributes to the pathogenesis of arthritis (17–19). For example, administration of N^G-monomethyl-L-arginine (a nonspecific inhibitor of NOS) or *N*-iminoethyl-L-lysine (a selective inhibitor of iNOS) at the time of adjuvant induction of arthritis profoundly reduced synovial inflammation, joint swelling, and tissue damage in rats (17–19). In light of the findings from the current study, it would be interesting to investigate whether glucosamine suppresses arthritis in human and animal models by inhibiting inducible NO synthesis. The use of glucosamine offers the following advantages over the competitive and noncompetitive inhibitors of iNOS. First, as an inhibitor of inducible NO synthesis and as a substrate for the synthesis of structural proteoglycans, glucosamine is unique in both preventing NO-mediated joint destruction and promoting

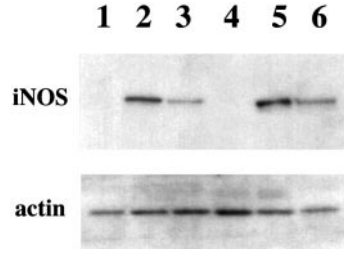

FIG. 2. Western blot analysis of iNOS in spleen and lung extracts. Extracts of lung and spleen from untreated rats (no LPS) and rats treated with LPS (1 mg/kg body wt) with or without glucosamine treatment (1.5 mmol/kg body wt/day) were immunoprecipitated with anti-iNOS antibody and subjected to Western blot analysis of iNOS. Extracts were also subjected to Western blot analysis of actin. Lanes 1–3 contain samples of lung and Lanes 4–6 contain samples of spleen. Lanes 1 and 4 contain extracts taken from untreated rats (no LPS), Lanes 2 and 5 contain extracts taken from LPS-treated rats, and Lanes 3 and 6 contain extracts from LPS- and glucosamine-treated rats.

the repair of joint cartilage. Second, glucosamine is a natural metabolite and has been shown to be safe for long-term administration to humans and animals (4–8). Oral administration of glucosamine to rats and dogs at doses up to 4.7 and 3.7 mmol/kg body wt/day for 12 and 6 months, respectively, does not have any adverse effect on the gastrointestinal tract or other organs (8). Third, by suppressing the expression of iNOS protein, glucosamine inhibits inducible NO synthesis independent of plasma arginine concentration (e.g., up to 10 mM [100 times normal levels] which can occur in patients receiving arginine infusion or total parenteral nutrition) (40, 41). High arginine concentrations can overcome the effect of competitive inhibitors of iNOS.

In conclusion, results of both *in vitro* and *in vivo* studies demonstrate that glucosamine is a novel inhibitor of inducible NO synthesis. Glucosamine inhibits inducible NO production by suppressing the expression of iNOS protein. Our findings provide a biochemical basis for the use of glucosamine in treating arthritis and may also have important implications for the prevention of the NO-mediated inflammatory diseases.

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