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Effect of inducible nitric oxide synthase inhibition by aminoguanidine on insulin-like growth factor binding protein-3 in adjuvant-induced arthritic rats

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Abstract

This study was designed to investigate whether nitric oxide (NO) mediates changes in insulin-like growth factor binding protein-3 (IGFBP-3) levels in rats with adjuvant-induced arthritis. Male Wistar rats were injected with complete Freund's adjuvant, and 20 days afterwards arthritic and control rats were injected daily with an inhibitor of inducible NO synthase (iNOS), aminoguanidine, or vehicle for 8 days. The increase in serum levels of IGFBP-3 induced by arthritis was exacerbated by aminoguanidine treatment. Arthritis increased IGFBP-3 mRNA levels in the kidney but not in the liver. The inhibition of iNOS did not modify IGFBP-3 gene expression in the kidney or in the liver in arthritic rats. However, the inhibitory effect of arthritis on the proteolysis of IGFBP-3 in serum was potentiated by aminoguanidine administration. These results indicate that arthritis increases serum IGFBP-3 by increasing its synthesis in the kidney and decreasing its proteolysis in serum and that these effects are not mediated by NO.

Keywords: iNOS [nitric oxide (NO) synthase, inducible]; Aminoguanidine; IGFBP-3 (insulin-like growth factor binding protein-3); IGFBP-3 proteolysis; IGFBP-3 mRNA; Arthritis, adjuvant-induced

1. Introduction

Adjuvant-induced arthritis is an animal model for chronic inflammation and rheumatoid arthritis in humans. Experimental arthritis is associated with a decrease in body weight and cachexia (Roubenoff et al., 1997), effects that are concomitant with endocrine modifications. Arthritis decreases both growth hormone secretion (Neidhart and Fluckiger, 1992; Selgas et al., 1997) and insulin-like growth factor-I (IGF-I) levels, and increases serum levels of IGF-binding protein-3 (IGFBP-3), the main binding protein that carries IGF-I in serum (López-Calderón et al., 1999). The high serum levels of IGFBP-3 in adjuvant arthritis are involved, at least in part, in the decrease in body weight, since normalization of IGFBP-3 serum levels in arthritic rats after growth hormone treatment increased body weight gain (Ibáñez de Cáceres et al., 2000).

The mechanism by which chronic arthritis increases the IGFBP-3 serum levels is not well characterised. Inhibition of its proteolysis in serum seems to be one mechanism involved in the increased IGFBP-3 serum levels in arthritic rats (Ibánez de Cáceres et al., 2002a). Furthermore, a decrease in IGFBP-3 proteolysis has also been described in the synovial fluid of patients with rheumatoid arthritis (Fernihough et al., 1996; Whellams et al., 2000). While the liver is the main source of serum IGFBP-3, the kidney has also been reported as another source (Feld and Hirschberg, 1996; Landau et al., 1995). In the liver of arthritic animals, IGFBP-3 mRNA levels are not modified, and therefore kidney-derived IGFBP-3 could contribute to the increased circulating IGFBP-3 levels in chronic arthritis.

The free radical nitric oxide (NO) is considered to be an important molecular mediator of numerous physiolog-

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ical processes such as neuronal communication and vascular tone (Cannon et al., 1996). NO is also a mediator of inflammatory and immunological diseases. The different properties of NO may vary according to its concentration. NO is produced by the action of the NO synthase (NOS) enzyme on L-arginine. There are three different isoforms of NOS, two of them produce NO in low concentrations and are constitutively expressed in neuronal (nNOS) and endothelial (eNOS) cells. The other is an inducible isoform (iNOS), which is mainly expressed by inflammatory cells following exposure to diverse stimuli. iNOS produces NO in high concentrations, which have been suggested to be toxic (Hernández-Pando et al., 2001).

Excessive NO synthesis by iNOS has been observed in experimental arthritis in rats (Cannon et al., 1996) and in patients with arthritis (Farrell et al., 1992; Sakurai et al., 1995). Cytokines such as interferon-γ, tumour necrosis factor-α and other inflammatory mediators can be involved in this process, since they induce iNOS expression in macrophages, hepatocytes and vascular smooth muscle cells. The NO generated by iNOS induction in macrophages following activation by either cytokines or endotoxin contributes to the cytotoxic and cytostatic properties of cytokines against target cells (Moncada et al., 1991).

In the kidney, NO plays a prominent role in the homeostatic regulation of glomerular, vascular, and tubular function (Kone and Baylis, 1997). However, NO overproduction by iNOS has been observed with the development of renal failure in several inflammatory illness, such as experimental glomerulonephritis (López-Franco et al., 2002), arteriosclerosis renovascular disease (Chade et al., 2002) and in sepsis after cytokine exposure (Blantz and Munger, 2002). An alteration in renal function has also been reported in arthritic rats (Dijoseph et al., 1993), and this alteration might be mediated by NO.

The aim of this work was to study IGFBP-3 synthesis in the kidney as a possible cause of the increased circulating IGFBP-3 levels in arthritic rats. The role of NO in the IGFBP-3 alterations described in arthritic rats was also analysed by administering aminoguanidine.

2. Materials and methods

2.1. Animals

Control and arthritic male Wistar rats were purchased from Charles River (Barcelona, Spain). They were housed three or four per cage under controlled conditions of temperature (20–22 °C) and light (lights on from 0730 to 1930 h). Food and water were made freely available. The procedures followed the guidelines recommended by the EU for the care and use of laboratory animals, and they were approved by the Institutional Ethics Committee.

2.2. Experimental design

Arthritis was induced by a single intradermal injection of complete Freund's adjuvant (1 mg of heat-killed *Mycobacterium butyricum*: Difco Laboratories, Detroit, MI, USA). The injection was given into the plantar surface of the right hind paw under ketamine (75 mg/kg) + diazepan (5 mg/kg) anaesthesia. Control rats were injected with vehicle (paraffin oil). Twenty days after adjuvant injection, control and arthritic animals were divided into two groups. One group was injected daily with 250 μl s.c. of phosphate-buffered saline (PBS) and the other group was injected s.c. daily with 200 mg/kg of aminoguanidine hemisulphate (Alexis, Switzerland). All groups of animals were injected for 8 days.

On day 28 after adjuvant injection, all rats were killed by decapitation 2.5 h after the last aminoguanidine or PBS injection. Trunk blood was allowed to clot and was centrifuged. Serum was stored at −20 °C until nitrite + nitrate and IGFBP-3 levels were measured and the proteolysis assay was performed. The spleen was removed and weighed. Liver and kidney were removed, dissected, frozen in liquid nitrogen and stored at −80 °C until RNA extraction was performed.

The arthritis index score of each animal was measured as previously described by Tanaka et al. (1996). The arthritis index of each animal was scored by grading each paw from 0 to 4. Grading was determined as: 0—no erythema or swelling; 1—slight erythema or swelling of one or more digits; 2—swelling of entire paw; 3—erythema and swelling of the ankle; 4—ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16.

2.3. Determination of nitrate and nitrite concentration

The serum was deproteinised to reduce turbidity by centrifugation through a 30-kDa molecular weight filter using a Centrifree Micropartition Device® with a YM-30 ultrafiltration membrane (Amicon Division, Millipor, Bedford, USA) at 1500 rpm for 1 h at 37 °C for 300-μl samples. Serum nitrate and nitrite concentration was measured by a modified method of Griess assay (Green et al., 1982) described by Miranda et al. (2001). One hundred microlitres of filtered serum was mixed with 100 μl of vanadium (III)-chloride, followed rapidly by the addition of Griess reagents. Thirty minutes later, the determination was performed at 37 °C. The absorbance was measured at 540 nm. Nitrate and nitrite concentration was calculated using a sodium nitrite standard curve.

2.4. Western ligand blot

Western blots were prepared as previously described by Hossenlopp et al. (1986). Two-microlitre aliquots of serum samples were diluted with sample buffer, boiled for 2 min at

100 °C, and then loaded onto 12.5% acrylamide gels containing 1% sodium dodecyl sulphate (SDS). Electrophoresis was performed under non-reducing conditions and proteins were transferred onto nitro-cellulose membranes (Hybond™-C extra, Amersham, Bucks, UK). The membranes were dried and blocked with 5% non-fat dry milk for 1 h, in Tris-buffered saline with 0.1% Tween-20 (Sigma, Madrid, Spain). Afterwards, the membranes were incubated overnight with [^{125}I] labelled IGF-I (5×10^5 cpm/ml) at 4 °C, and the blots were exposed at −80 °C to X-ray film (Kodak X-Omat AR, Eastman Kodak, Rochester, NY, USA) for 2–4 days.

2.5. RNA extraction and Northern blot analysis of IGFBP-3 mRNA content

Total RNA from liver and kidney was extracted by the guanidine thiocyanate method using a commercial reagent (Ultraspec™ RNA, Biotecx Laboratories, Houston, TX, USA). The extracted RNA was dissolved in diethyl pyrocarbonate water with 0.1% SDS and quantified at 260 nm; RNA integrity was confirmed by agarose gel electrophoresis. For Northern blotting, 40 μg of denatured RNA from either liver or kidney was separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes (Hybond-N+, Amersham, UK) and fixed by UV cross linking (Fotodyne Hartland, WI, USA). For the rat IGFBP-3 probe, a DNA fragment of rat cDNA (Albiston and Herington, 1990) was inserted into the pGEM-4Z plasmid vector. The insert was released by *Eco*RI and *Hin*dIII cleavage and was labelled with [^{32}P] dCTP (Nucliber, Madrid, Spain) by using the random priming DNA labelling kit (DECAprime™ II, Ambion, Austin, TX, USA). Prehybridisation was performed for 30 min at 42 °C in ULTRAhyb™ buffer (Ambion), followed by hybridisation for 16 h at the same temperature with 3×10^6 cpm/ml IGFBP-3 labelled probe. The membranes were washed twice with 2 × sodium salt citrate (SSC) buffer (3 M NaCl, 0.3 M Na_3 citrate × 2 H_2O, pH 7.0), 0.1% SDS at 42 °C for 10 min, and twice with 0.1% × SSC, 0.1% SDS for the same time and temperature. X-ray film was exposed at −80 °C for 2–7 days. Homogeneity of gel loading was confirmed by the intensity of the ribosomal 28S RNA bands in the transferred membranes reprobed with 28S cDNA labelled with [^{32}P] dCTP.

2.6. Assay for IGFBP-3 proteolytic activity

Protease activity against IGFBP-3 in serum samples was detected as previously described (Ibánez de Cáceres et al., 2002a). Recombinant human glycosylated IGFBP-3 (GroPep, Adelaide, Australia) was iodinated using the chloramine T method. Serum samples (5 μl) were mixed with 15,000 cpm [^{125}I] gIGFBP-3 in a total volume of 30 μl in 0.05 M phosphate buffer pH 7.5. The mixture was incubated for 18 h at 37 °C and the reaction was stopped by boiling the mixture with 7 μl (5 ×) SDS loading buffer before loading onto 12.5% acrylamide gels containing 1% SDS. Following electrophoresis, gels were stained in Coomassie brilliant blue and labelled substrates were visualised after fixing and drying the gels (Biorad gel drying system-543) on X-ray film after exposure at −80 °C for 2–3 days.

2.7. Quantification of Western ligand blot, IGFBP-3 degradation assay and Northern blot

The intensities of autoradiograph signal levels were analysed by densitometric scanning using a PC-Image (Foster Findlay Associates, Newcastle, UK) VGA24 program for Windows.

In Western ligand blotting, the density of the IGFBP-3 band in each lane of the autoradiograph is expressed as the percentage of the mean density of sera from control rats injected with PBS.

In the proteolysis assay, the intensity of the band is negatively correlated with the proteolytic activity, so band intensity decreases (less [^{125}I]IGFBP-3) as proteolytic activity increases. The amount of proteolysis is expressed as the percentage decrease in [^{125}I]IGFBP-3 in each serum sample relative to the [^{125}I]IGFBP-3 incubated with heat-inactivated serum (0% of proteolysis).

In Northern blot analysis, the hybridisation signals of the IGFBP-3 band were normalised to that of 28S in each sample and expressed as the percentage of the mean intensity of the control group injected with PBS.

2.8. Statistical analysis

Statistics were analysed using the program STATGRAPHICS plus for Windows. Data are presented as means ± S.E.M. and analysed with a multifactorial analysis of variance (ANOVA) with arthritis and treatment with aminoguanidine as factors. Individual means were com-

Table 1
Effect of arthritis and aminoguanidine (AG) treatment on serum NO_x levels, on arthritis index scores, and on relative spleen weight

	Serum NO_x (μM)	Arthritis scores	Spleen (mg/100 g bw)
Control + PBS	28.00 ± 1.17		309.43 ± 11.53
Control + AG	22.70 ± 1.49		310.42 ± 12.84
Arthritic + PBS	57.52 ± 3.97[a]	12.00 ± 1.12	752.30 ± 58.83[a]
Arthritic + AG	40.20 ± 3.70[a,b]	8.78 ± 0.83[b]	615.90 ± 42.46[a,c]

Arthritis was induced by injection of *M. butyricum* into the plantar surface of the right hind paw on day 0. Control rats were injected with vehicle (paraffin oil). Aminoguanidine or PBS was administered daily from days 20 to 28. Values are the means ± S.E.M. for 10 rats in each group.

[a] $P < 0.01$ vs. with control group injected with PBS (Duncan's multiple range test).

[b] $P < 0.01$ vs. with arthritic group injected with PBS (Duncan's multiple range test or Student's *t*-test).

[c] $P < 0.05$ vs. with arthritic group injected with PBS (Duncan's multiple range test or Student's *t*-test).

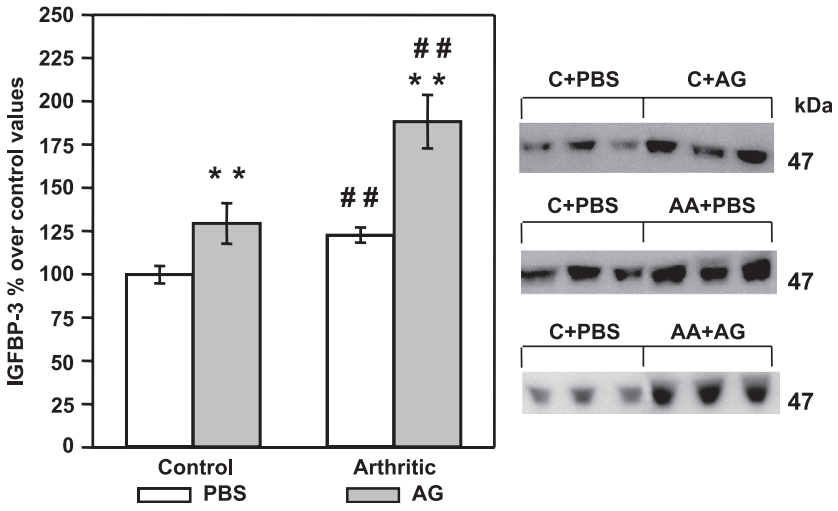

Fig. 1. Effect of aminoguanidine (AG) administration on serum IGF-I-binding activity of IGFBP-3. Representative Western ligand blots of serum IGFBP-3 from the different groups are shown on the right. IGFBPs were separated by SDS-PAGE, transferred to nitrocellulose, ligand blotted with [^{125}I] IGF-I, and visualised via autoradiography; approximate molecular weight of the band is indicated on the right. C + PBS = control rats injected with PBS, C + AG = control rats treated with aminoguanidine, AA + PBS = arthritic rats injected with PBS, AA + AG = arthritic rats treated with aminoguanidine. On the left, data from 10 individual rats were quantified by densitometry and expressed as percentage of the mean value of control rats treated with PBS (means ± S.E.M.) ##$P < 0.01$ vs. the respective control group, **$P < 0.01$ vs. the respective group injected with PBS. ANOVA test. There were no significant interactions between the two factors, arthritis and aminoguanidine treatment.

pared by Duncan's multiple range test. Statistical significance was assumed at $P < 0.05$. Comparisons between two groups were performed using Student's *t*-test.

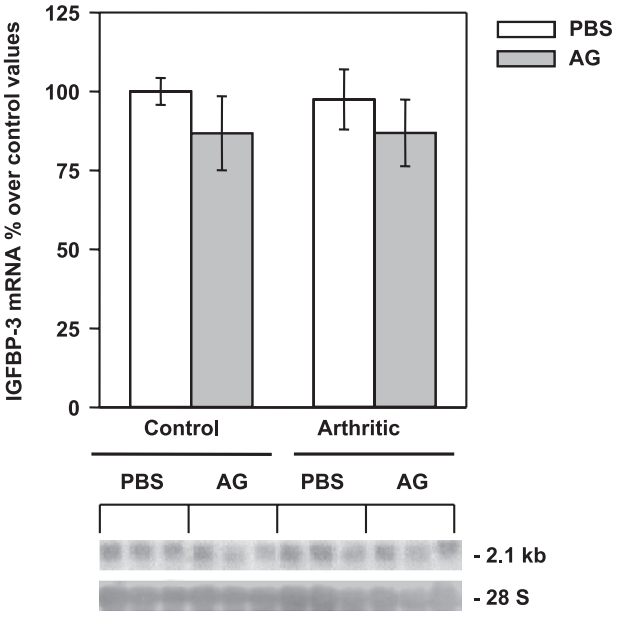

Fig. 2. Effect of adjuvant-induced arthritis and aminoguanidine (AG) administration on liver IGFBP-3 mRNA content. A representative Northern blot of IGFBP-3 mRNA hybridisation is shown in the lower panel. RNA was hybridised with a cDNA probe for rat IGFBP-3 mRNA as described in Materials and methods. The size of the hybridisation band (in kilobases) and ribosomal 28S RNA are indicated on the right; each line corresponds to an individual animal from the indicated group. Quantitative analyses are expressed as percentages of data for control rats injected with PBS (upper panel). ANOVA + Duncan multiple comparison test. Each bar represents the mean ± S.E.M. for 10 rats.

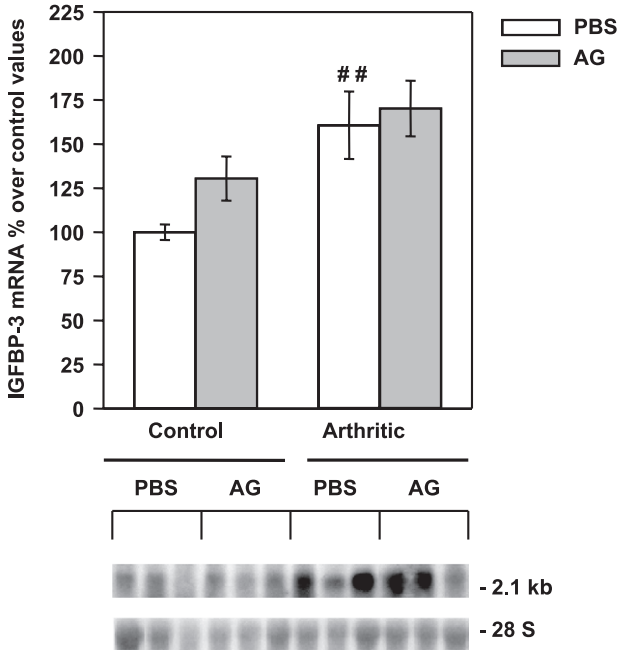

Fig. 3. Effect of adjuvant-induced arthritis and aminoguanidine (AG) administration on kidney IGFBP-3 mRNA. A representative Northern blot of IGFBP-3 mRNA hybridisation is shown in the lower panel. RNA was hybridised with a cDNA probe for rat IGFBP-3 mRNA as described in Materials and methods. The size of the hybridisation band (in kilobases) and ribosomal 28S RNA are indicated on the right; each line corresponds to an individual animal from the indicated group. Densitometric measurements of the bands are shown as a percentage of values for control rats injected with PBS (upper panel). ANOVA + Duncan multiple comparison test. Data represent the means ± S.E.M. for 10 rats. ##$P < 0.01$ vs. control group injected with PBS.

3. Results

As expected, arthritis significantly increased ($P<0.01$) serum nitrate + nitrite levels and aminoguanidine administration decreased them (Table 1). The increase in the relative spleen weight induced by arthritis was reduced by aminoguanidine administration ($P<0.05$). Moreover, the arthritis index scores were significantly decreased after 8 days of treatment with the iNOS inhibitor (8.78 ± 0.83 vs. 12.00 ± 1.12 in arthritic rats treated with PBS; $P<0.01$). Treatment with aminoguanidine did not modify body weight in either control or arthritic rats (data not shown).

Adjuvant-induced arthritis increased IGFBP-3 serum levels ($P<0.01$) and aminoguanidine administration raised the IGFBP-3 serum level both in control and in arthritic rats ($P<0.01$) (Fig. 1). There was no interaction between the two factors, arthritis and aminoguanidine treatment. Therefore, aminoguanidine treatment had similar effects, increasing the serum IGFBP-3 levels, in both control and arthritic rats.

The increase in IGFBP-3 serum levels induced by arthritis or by iNOS inhibition with aminoguanidine does not seem to be secondary to an increase in its hepatic synthesis. As can be seen in Fig. 2, liver IGFBP-3 mRNA expression was not modified by arthritis or by aminoguanidine treatment.

In contrast, arthritis increased IGFBP-3 gene expression in the kidney ($P<0.01$) (Fig. 3). But, similarly to data obtained for the hepatic synthesis of IGFBP-3, the inhibition of iNOS by aminoguanidine administration did not modify the renal IGFBP-3 mRNA levels in control or in arthritic rats.

IGFBP-3 proteolytic activity in the serum of control and arthritic rats after PBS or aminoguanidine administration is shown in Fig. 4. Proteolysis of IGFBP-3 was decreased in the serum of arthritic rats. In addition, the inhibition of iNOS by aminoguanidine treatment also reduced IGFBP-3 proteolysis in the serum of both control and arthritic rats.

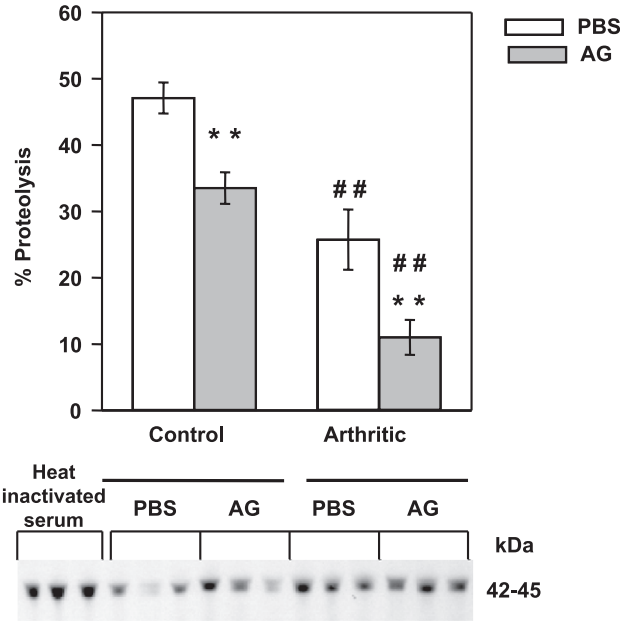

Fig. 4. IGFBP-3 proteolytic activity in serum of control or arthritic rats treated with PBS or aminoguanidine for 8 days. [^{125}I] hIGFBP-3 was incubated 18 h at 37 °C with sera and submitted to SDS-PAGE as described in Materials and methods. A representative autoradiograph of IGFBP-3 protease assay of rat serum samples from control and arthritic groups treated with PBS or aminoguanidine (AG) and heat-inactivated serum are shown in the lower panel. Heat-inactivated serum was used as a control, as no proteolytic activity is found in the serum after being heated. Molecular weight is shown on the right. IGFBP-3 proteolysis was expressed as percent decrease in [^{125}I] hIGFBP-3 in each sample relative to [^{125}I] hIGFBP-3 in control samples incubated with heat-inactivated serum (upper panel). Each point represents the mean ± S.E.M. for 10 rats per group. ##$P<0.01$ vs. the respective control group, **$P<0.01$ vs. the respective group injected with PBS. ANOVA test. There was no significant interaction between the two factors, arthritis and treatment with aminoguanidine.

4. Discussion

In this study, control and arthritic animals were injected with an inhibitor of iNOS activity, aminoguanidine, to study the role of nitric oxide on IGFBP-3 regulation in experimental arthritis. The results of this study indicate on one hand, the different regulation of IGFBP-3 gene expression in the liver and in the kidney in experimental arthritis and on the other, that NO is not involved in the IGFBP-3 alterations in arthritic rats.

The effect of aminoguanidine treatment in decreasing NO concentrations in arthritic animals has been previously described with a different dose and route of administration (Gad and Khattab, 2000). In the present study, aminoguanidine at a dose of 200 mg/kg was effective in reducing NO production in arthritic rats, as serum nitrite + nitrate concentration was lower than that found in arthritic rats treated with PBS. In the present study, the treatment with aminoguanidine also had therapeutic effects in rats with established arthritis. Tanaka et al. (1998) found that prophylactic administration of aminoguanidine for 18 days at the same dose and by the same route of administration as used in this study also suppressed paw swelling and splenomegaly in arthritic rats. However, other authors (Stefanovic-Racic et al., 1995) did not find any improvement in the paw swelling of arthritic rats after prophylactic or therapeutic administration of aminoguanidine. In the present study, the effect of aminoguanidine on the arthritis index scores and splenomegaly in arthritic rats seems to be due to its action as a specific iNOS inhibitor. However, aminoguanidine has other functions besides iNOS inhibition. For example, it is used to prevent the formation of glycated protein (Skamarauskas et al., 1996) and is also an inhibitor of amino oxidase activity (Yu and Zuo, 1997), and these actions reveal a potential use of aminoguanidine as a prophylactic agent in diabetes mellitus.

This is the first study in which the levels of IGFBP-3 mRNA were measured in the kidney of arthritic rats, and

the results indicate that adjuvant-induced arthritis results in an increase in IGFBP-3 gene expression in the kidney; however, there is not any significant change in IGFBP-3 mRNA levels in the liver. Similarly, our group has not reported any alteration in liver IGFBP-3 gene expression in arthritic rats on day 22 after adjuvant injection (Ibánez de Cáceres et al., 2002a). These findings indicate that IGFBP-3 synthesis in adjuvant-induced arthritis seems to be regulated differently in the liver and in the kidney. This result is in contrast with that for IGF-I gene expression, where similar changes were detected in both tissues in rats with adjuvant-induced arthritis. However, differences between liver and kidney have been described in other components of the somatotropic axis in arthritis, such as the levels of IGF-I protein, which are lower in the liver, while in the kidney the IGF-I concentration is higher (Ibánez de Cáceres et al., 2002b). Thus, the increase in IGFBP-3 synthesis in the kidney of arthritic rats could explain the high levels of IGF-I detected in the kidney of adjuvant-induced arthritic rats, since IGFBP-3 could trap circulating IGF-I in this tissue.

Arthritis increased the IGFBP-3 serum levels, as has been previously reported in rats with experimental arthritis (López-Calderón et al., 1999) and in humans with rheumatoid arthritis (Neidel, 2001). Although the liver is believed to be the main source of circulating IGFBP-3, its gene expression is not modified in arthritis. Therefore, the high serum levels of IGFBP-3 in arthritic rats must come from a different source than the liver. The kidney has been previously reported as a source of circulating IGFBP-3 (Feld and Hirschberg, 1996; Landau et al., 1995), and the increased synthesis of IGFBP-3 in the kidney of arthritic rats may be involved, at least in part, in the higher levels of serum IGFBP-3 in this group of animals. However, adjuvant-induced arthritis decreased IGFBP-3 proteolysis in serum (Ibánez de Cáceres et al., 2002a). These two findings may underlie the increased IGFBP-3 serum levels in the arthritic rats.

The inhibition of iNOS activity did not produce any change in the levels of IGFBP-3 mRNA in liver and kidney or in control and arthritic rats. These findings confirm the results of Iñiguez et al. (2001), who described that the administration of the NO synthase substrate, L-arginine, did not modify the IGFBP-3 production of human corpora lutea. These results indicate that NO does not seem to be involved in the regulation of IGFBP-3 gene expression. The increase in the synthesis of IGFBP-3 in the kidney of arthritic rats could be mediated by other inflammatory agents apart from NO. Cytokines could be involved in this process, as their levels are increased in arthritic rats. It has also been reported that interleukin-1 and tumour necrosis factor-α increase IGFBP-3 production in chondrocytes in several models of inflammatory joint disease (Olney et al., 1995). In contrast to these results, it has been described that the administration of tumour necrosis factor-α to rats reduces IGFBP-3 mRNA expression in kidney (Lang et al., 2001). The discrepancy between these studies could be due to the different experimental models used. The first study used articular chondrocytes in primary culture, whereas the second study used conscious rats intravenously infused with tumour necrosis factor-α.

Although it seems that NO is not involved in the regulation of IGFBP-3 gene expression, it does seem to have a principal role in regulating the IGFBP-3 level in serum, as its inhibition with aminoguanidine treatment produced an increase in IGFBP-3 serum level in both control and arthritic rats. Our findings also show that NO activates IGFBP-3 serum proteolysis, as the inhibition of iNOS by aminoguanidine treatment induced a decrease in IGFBP-3 serum fragmentation in both control and arthritic groups. This inhibition of serum proteolytic activity occurred concomitantly with the described increase in IGFBP-3 serum levels in both groups of rats treated with aminoguanidine.

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