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S-Substituted isothioureas are potent inhibitors of nitric oxide biosynthesis in cartilage

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Abstract

Nitric oxide (NO·) is a multifunctional messenger molecule generated by a family of enzymes, the nitric oxide synthases, and is overproduced in osteoarthritis and rheumatoid arthritis. Chondrocytes are the major native source of NO· in diarthrodial joints. Chondrocytic inducible nitric oxide synthase induced by inflammatory cytokines and bacterial cell wall fragments mediates many of the catabolic events in arthritis. Agents which specifically inhibit chondrocyte inducible NO· synthase, may thus have a role in the management in arthritis. We evaluated a novel class of potential inducible NO· synthase inhibitors, the *S*-substituted isothioureas, for their ability to inhibit inducible NO· synthase activity in cultured bovine chondrocytes and explants of cartilage from patients with osteoarthritis. Two isothioureas, *S*-methyl isothiourea and *S*-(aminoethyl) isothiourea were 2–4 times more potent than N^G-monomethyl-L-arginine monoacetate, 5–10 times more potent than aminoguanidine and over 300 times more potent than N^ω-nitro-L-arginine and N^ω-nitro-L-arginine methyl ester. The rank order of potency of the NO· synthase inhibitors was *S*-(aminoethyl) isothiourea > *S*-methyl isothiourea > N^G-monomethyl-L-arginine > aminoguanidine > N^ω-nitro-L-arginine = N^ω-nitro-L-arginine methyl ester. The order of potency was reversed (N^ω-nitro-L-arginine methyl ester = N^ω-nitro-L-arginine > N^G-monomethyl-L-arginine = *S*-methyl isothiourea > *S*-(aminoethyl) isothiourea > aminoguanidine) when evaluating the same compounds ability to inhibit constitutive NO· synthase activity in bovine endothelial cells. In comparison to conventional arginine-based analogs, the isothioureas represent a more potent and relatively specific class of inhibitors of inducible NO· synthase in cartilage and thus may be beneficial in the management of arthritis.

Keywords: Cartilage; Chondrocyte; Nitric oxide (NO) synthase, inducible; Free radical; Arthritis

1. Introduction

Nitric oxide (NO·) is formed from L-arginine by a family of enzymes, the NO· synthases, which oxidize one of the guanidino nitrogen atoms in L-arginine to form L-citrulline and NO·. Three isoforms of nitric oxide synthase (NO· synthase) have been cloned and sequenced. Endothelial cell NO· synthase and brain NO· synthase are constitutively active. The inducible isoform of NO· synthase is found in a variety of cells including macrophages (Hevel et al., 1991; Hibbs et al., 1988), neutrophils, lymphocytes (Salvemini et al., 1990), hepatocytes (Chartrain et al., 1994) and chondrocytes (Stadler et al., 1991). The inducible NO· synthase is absent in these cells under basal conditions, but can be induced by bacterial cell wall products (e.g. endotoxin and staphylococcal factor), and by inflammatory mediators, such as interleukin-1β and tumor necrosis factor-α. Once induced, inducible NO· synthase produces large amounts of NO· for prolonged periods, which plays an important role in host defense (Nathan and Hibbs, 1991). When overproduced, however, NO· may contribute to pathophysiological processes, including septic shock (Szabo, 1995), inflammatory diseases of the gut wall (Miller et al., 1993), and diarthrodial joints (Farrell et al., 1992).

In arthritis, the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α, modulate the biochemical pathways that are characteristic of cartilage destruction: activation of the prostaglandin pathway via cyclooxygenase (Salvemini et al., 1993) decreased synthesis of major components of the cartilage matrix, the proteoglycans (Taskiran et al., 1994) and type II collagen (Goldring

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et al., 1988), and increased activity of the metal-dependent enzymes that degrade this matrix (collagenase, gelatinase and stromelysin). Data from our (Manfield et al., 1996; Murrell et al., 1995a,b), and other laboratories (Hickery et al., 1994; Taskiran et al., 1994), suggest that these pathways are connected by inducible NO· synthase and that L-arginine-NO· pathway mediates many of the catabolic effects of the inflammatory cytokines in cartilage. This pathway, therefore, offers a particularly attractive target for the pharmacological inhibition of cartilage degradation in osteoarthritis and other inflammatory-mediated destructive arthropathies. As NO· is such an important and ubiquitous messenger molecule, and as chondrocytes are the major native source of NO· in inflamed diarthrodial joints (Murrell et al., 1995a; Rediske et al., 1994) NO· synthase inhibitors need to be specific for chondrocyte inducible NO· synthase. The aim of this investigation, therefore, was to evaluate the ability of a novel class of NO· synthase inhibitors, the *S*-substituted isothioureas (Garvey et al., 1994; Nakane et al., 1995; Southan et al., 1995; Szabo et al., 1994), to inhibit chondrocyte inducible NO· synthase activity.

2. Materials and methods

2.1. Materials

N^G-Monomethyl-L-arginine monoacetate(MeArg) was from Calbiochem, San Diego, CA, USA. Human recombinant tumor necrosis factor-α was from R&D Systems, Minneapolis, MN, USA. *S*-(2-aminoethyl) isothiouronium bromide hydrobromide (AETU), *S*-methyl isothiourea sulphate (SMT) and N^{ω}-nitro-L-arginine (NO_2Arg) were from Aldrich Chemical, Milwaukee, WI, USA. All other biochemicals including lipopolysaccharide, aminoguanidine bicarbonate salt, N^{ω}-nitro-L-arginine methyl ester (NO_2-ArgME) and L-arginine were purchased from Sigma Chemical, St. Louis, MO, USA. Cell culture reagents were from Gibco Laboratories, New York, NY, USA.

2.2. Chondrocyte isolation

Slices of occipital cartilage from freshly slaughtered cows were digested to obtain chondrocytes and cultured under conditions which support the maintenance of the chondrocyte phenotype (Green, 1971). Briefly, the bovine tissue was vigorously washed with 10% antibiotic-antimycotic in normal saline. The tissue was diced in an appliance blender and incubated in 0.025% (w/v) collagenase, 1% (v/v) antibiotic-antimycotic, 2% (v/v) Hepes at 37°C for 24 h under gentle agitation. The cells were recovered, washed with 10% antibiotic-antimycotic in Dulbecco's modified Eagle medium (DMEM) for 20 min and, centrifuged at $2000 \times g$. The pellet was suspended and plated with DMEM + 10% fetal calf serum (FCS) in 75 ml culture flasks at 2×10^6 cells/flask. Confluent chondrocyte flasks were plated in 96-well culture plates at a density of 10^5 cells/well in 200 μl DMEM + 10% FCS and allowed to adhere overnight. The following day, the medium was replaced with serum-free DMEM and cells were incubated for 24 h under varying experimental conditions (final well volume = 250 μl).

2.3. Cartilage explant assays

Slices of occipital cartilage from freshly slaughtered cows were washed with 10% antibiotic-antimycotic in normal saline. Under sterile conditions, the cartilage was fashioned into 4 mm diameter discs approximately 2 mm in thickness using a 4 mm biopsy punch.

Human cartilage was obtained from patients with osteoarthritis undergoing total knee replacements. Under sterile conditions, cuts of bone and cartilage taken from the tibial plateau and femoral condyles during the total knee replacement were cleared of bone, and the cartilage fashioned into 4 mm diameter plugs with a biopsy punch. All tissue explants were then subjected to the same experimental procedure as the bovine chondrocytes.

2.4. Nitrite determination

Nitrite, the stable end-product of NO was measured via the colorimetric Greiss reaction from conditioned media, and the absorbance read on a 340 ATTC spectrophotometer (Tecan US, Research Triangle Park, NC, USA) at 550/650 nm. Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in DMEM.

Table 1
Isothiourea inhibition of nitric oxide synthase is reversed by exogenous L-arginine

Inhibitor		Nitrite (% of control)	
		L-Arginine	
		0.4 (mM)	1.8 (mM)
AETU	-3	4±0.4	7±0
	-5	58±1	77±0.5
	-7	100±2	109±0.8
MeArg	-3	8±0.4	62±0.4
	-5	81±1	114±0.5
	-7	89±3	104±0.4

Cultured bovine chondrocytes were stimulated by 10 μg/ml lipopolysaccharide in the presence of varying concentrations of the NO· synthase inhibitors, *S*-(2-aminoethyl)isothiourea (AETU) and the reference compound N^G-monomethyl-L-arginine monoacetate (MeArg). Inhibition of nitrite formation was reversed by the addition of exogenous L-arginine, the substrate for NO· synthase. Expressed as mean percentages ± S.E.M. of stimulated control; mean nitrite accumulation for control groups in 0.4 mM L-arginine = 39 nmol nitrite/10^6 cells/24 h, for 1.8 mM L-arginine = 66 and 70 nmol nitrite/10^6 cells/24 h for AETU and MeArg, respectively, $n = 6$ for each group.

2.5. Cell viability

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Cells or explants in 96-well plates were incubated with MTT (0.4 mg/ml) in DMEM for 60 min. Cell monolayer viability was assessed by

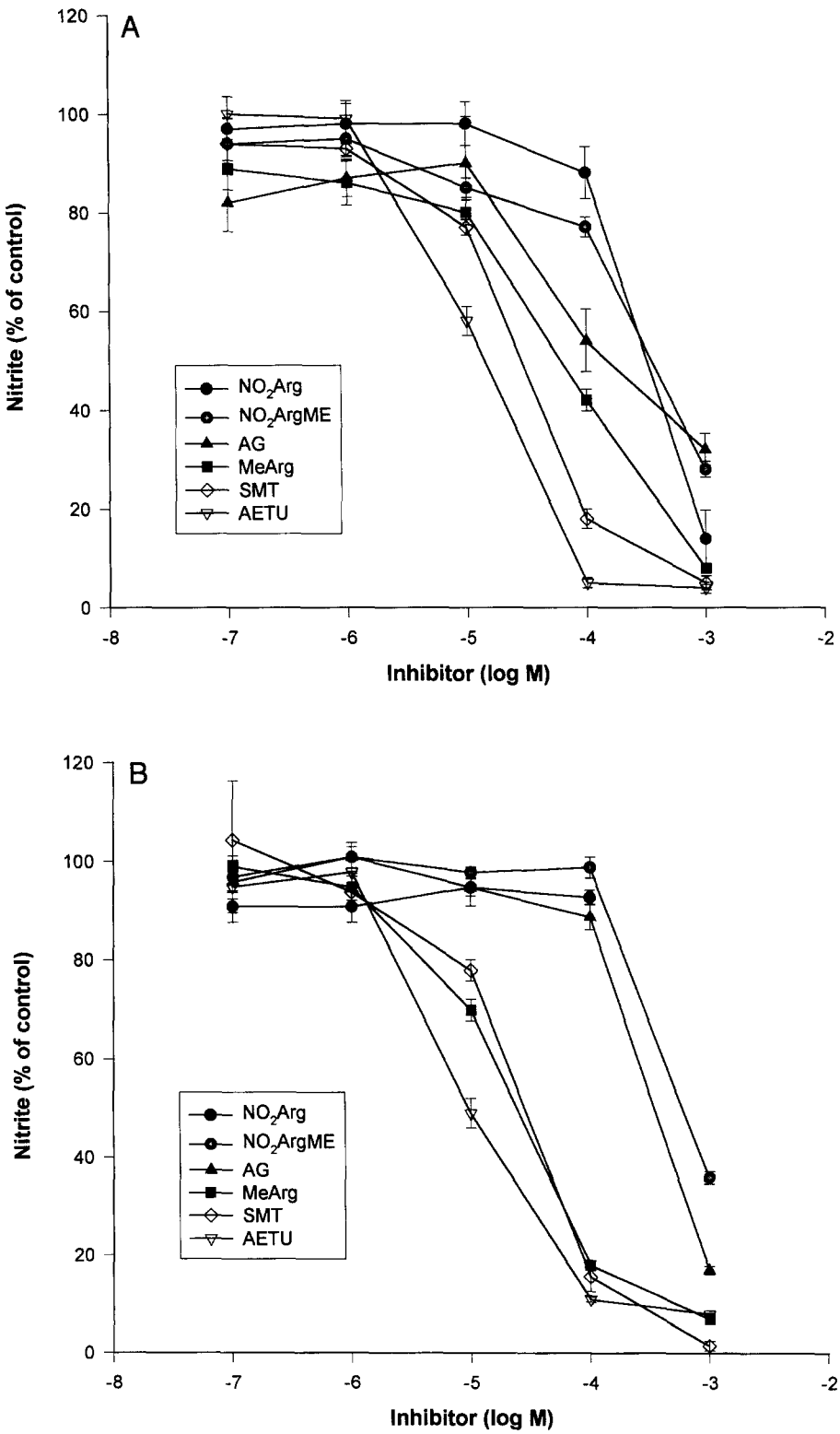

Fig. 1. Effect of N^{ω}-nitro-L-arginine (NO_2Arg), N^{ω}-nitro-L-arginine methyl ester (NO_2ArgME), aminoguanidine (AG), N^{G}-monomethyl-L-arginine monoacetate (MeArg), *S*-methyl isothiourea (SMT) and *S*-(aminoethyl) isothiourea (AETU) on the increase in nitrite concentration in the media of cultured bovine chondrocytes activated with either 10 μg/ml lipopolysaccharide (a) or 100 ng/ml tumor necrosis factor-α (b) for 24 h. Expressed as means ± S.E.M. of stimulated control. Mean value for control group in (a) 39 nmol nitrite/10^6 cells/24 h, for (b) 80 nmol nitrite/10^6 cells/24 h.

solubilizing the cells in dimethyl sulfoxide (200 μl). The extent of reduction of MTT to formazan within cells was quantified by measurement of absorbance at 550/650 nm. Formazan production was expressed as a percentage of that observed in the control group. Explant viability was evaluated by gross examination. Healthy cartilage turned blue whereas dead tissue did not change color. Results are only expressed when there was no significant reduction of formazan formation.

2.6. Selectivity studies: effect of NO· synthase inhibitors on nitrite accumulation in immuno-stimulated macrophages and on citrulline formation by bovine endothelial cell homogenates

The mouse macrophage cell line J774.2 was cultured in DMEM with 4×10^{-3} M L-glutamine and 10% FCS as described (Szabo et al., 1994). Cells were cultured in 96-well plates to subconfluence. To induce inducible NO· synthase, serume-free medium containing lipopolysaccharide (10 μg/ml) and murine interferon-γ (50 U/ml) was added. Nitrite accumulation in the cell culture medium (in the absence or presence of various inhibitors) was measured by the Greiss reaction after 24 h. Results shown represent the means of data obtained in $n = 6–12$ wells.

Ca^{2+}-dependent conversion of L-arginine to L-citrulline in cell homogenates obtained from the scraped intimal surface of fresh bovine aortae served as a model of endothelial NO· synthase activity (Southan et al., 1995). Cells were homogenized in a buffer composed of 50 mM Tris-HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) on ice using a Tissue Tearor 985-370 homogenizer (Biospec Products, Racine, WI, USA). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates as described (Southan et al., 1995). Briefly, cell homogenate (30 μl) was incubated in the presence of [^{3}H]L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and Ca^{2+} (2 mM) for 20 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice cold Hepes buffer (pH 5.5) containing EGTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]L-citrulline activity measured by a Wallac scintillation counter (Wallac, Gaithersberg, MD, USA). Endothelial NO· synthase in the cell homogenates was 1.8 ± 0.1 pmol/mg/min. Results shown represent means of data of $n = 6$ determinations.

2.7. Statistical comparison

All values in the text and figures are expressed as means ± standard error of the mean of n observations. Statistical analysis between experimental groups was performed using unpaired two-tailed Student's t-tests. The confidence limit was predetermined at an α level of 0.05.

3. Results

3.1. Isothiourea inhibition of nitric oxide synthase is reversed by exogenous L-arginine

Cultured bovine chondrocytes and explants of human cartilage released nitrite, the stable end-product of NO·, when stimulated by 1–100 ng/ml of the inflammatory cytokines tumor necrosis factor-α and interleukin-1β or 1–100 μg/ml of bacterial lipopolysaccharide. The nitrite production was time- (minimal at 6 h, maximal at 24 h) and cytokine dose-dependent (not shown). Nitrite production by inflammatory cytokine-induced cultured chondrocytes and cartilage was inhibited by all the NO· synthase inhibitors tested: N^{ω}-nitro-L-arginine, N^{ω}-nitro-L-arginine methyl ester, aminoguanidine, N^{G}-monomethyl-L-arginine monoacetate, *S*-methyl isothiourea and *S*-(aminoethyl) isothiourea. The inhibitory effects on nitrite formation by all compounds tested in cultured bovine chondrocytes were

Table 2
Comparison of the effective concentration at which half maximal inhibition occurred (EC_{50}) for inducible nitric oxide synthase (iNOS) in bovine chondrocytes, human cartilage, mouse macrophages and for constitutive nitric oxide synthase (cNOS) in bovine endothelial cells

Inhibitor	EC_{50} (μM)					
	Cultured bovine chondrocytes (iNOS)		Human osteoarthritic cartilage (iNOS)		Murine macrophages (iNOS)	Endothelial cell homogenates (cNOS)
	TNF-α	LPS	TNF-α	LPS	LPS + IFN	
AETU	9	15	9	30	6	50
SMT	28	30	–	–	10	5
MeArg	23	60	30	110	160	5
AG	350	150	–	–	120	2600
NO_2Arg	–	320		–	> 1000	1
NO_2ArgME	600	350	> 1000	190	> 1000	1

Cells or explants were incubated in the presence or absence of 100 ng/ml tumor necrosis factor-α (TNF-α) or 10 μg/ml lipopolysaccharide (LPS) and γ-interferon (IFN) for 48 h with nitric oxide synthase inhibitors: *S*-(2-aminoethyl) isothiourea (AETU), *S*-methyl isothiourea (SMT), N^{G}-monomethyl-L-arginine monoacetate (MeArg), aminoguanidine (AG), N^{ω}-nitro-L-arginine (NO_2Arg), N^{ω}-nitro-L-arginine methyl ester (NO_2ArgME).

reversed by the addition of exogenous L-arginine (Table 1), suggesting that these compounds competitively inhibit nitric oxide biosynthesis.

3.2. Isothioureas are potent inhibitors of nitric oxide synthase in cultured chondrocytes

N^{ω}-Nitro-L-arginine, N^{ω}-nitro-L-arginine methyl ester, aminoguanidine, N^{G}-monomethyl-L-arginine monoacetate, *S*-methyl isothiourea and *S*-(aminoethyl) isothiourea all inhibited nitrite production in lipopolysaccharide-induced cultured bovine chondrocytes in a dose-dependent manner (Fig. 1). *S*-(Aminoethyl) isothiourea and *S*-methyl isothiourea were the most potent inhibitors in this system. The effective concentration at which half maximal inhibition occurred (EC_{50}) of *S*-methyl isothiourea and *S*-(aminoethyl) isothiourea was 2–4 times lower than N^{G}-monomethyl-L-arginine monoacetate and 5–10 times lower than aminoguanidine. The EC_{50} values for *S*-(aminoethyl) isothiourea and *S*-methyl isothiourea were over ten times lower than N^{ω}-nitro-L-arginine and N^{ω}-nitro-L-arginine methyl ester in both lipopolysaccharide and tumor necrosis factor-α-stimulated chondrocytes (Table 2). When comparing the two isothioureas, *S*-(aminoethyl) isothiourea was the more potent, having EC_{50} values of 9 and 15 in tumor necrosis factor-α and lipopolysaccharide-induced chondrocytes, compared with EC_{50} values of 28 and 30 for *S*-methyl isothiourea. Thus, the rank order of potency on inducible NO· synthase inhibitors in cultured bovine chondrocytes, from most to least potent, was *S*-(aminoethyl) isothiourea > *S*-methyl isothiourea > N^{G}-monomethyl-L-arginine > aminoguanidine > N^{ω}-nitro-L-arginine = N^{ω}-nitro-L-arginine methyl ester.

3.3. Isothioureas potently inhibit inducible nitric oxide synthase in human cartilage

N^{ω}-Nitro-L-arginine methyl ester, N^{G}-monomethyl-L-arginine monoacetate and *S*-(aminoethyl) isothiourea all inhibited nitrite production in tumor necrosis factor-α and lipopolysaccharide-induced explants of human osteoarthritic cartilage in a dose-dependent manner (Fig. 2). *S*-(Aminoethyl) isothiourea was the most potent inhibitor in this system, with an EC_{50} 3–4 times lower than N^{G}-monomethyl-L-arginine monoacetate and 6–100 times lower than N^{ω}-nitro-L-arginine methyl ester. Thus, the rank order of potency on inducible NO· synthase inhibitors in cultured human cartilage, from most to least potent, was *S*-(aminoethyl) isothiourea > N^{G}-monomethyl-L-arginine > N^{ω}-nitro-L-arginine methyl ester.

3.4. Selectivity of isothioureas

The rank order of potency of inhibition of nitrite production of immuno-stimulated macrophages was similar to that seen in the immuno-stimulated chondrocytes, being

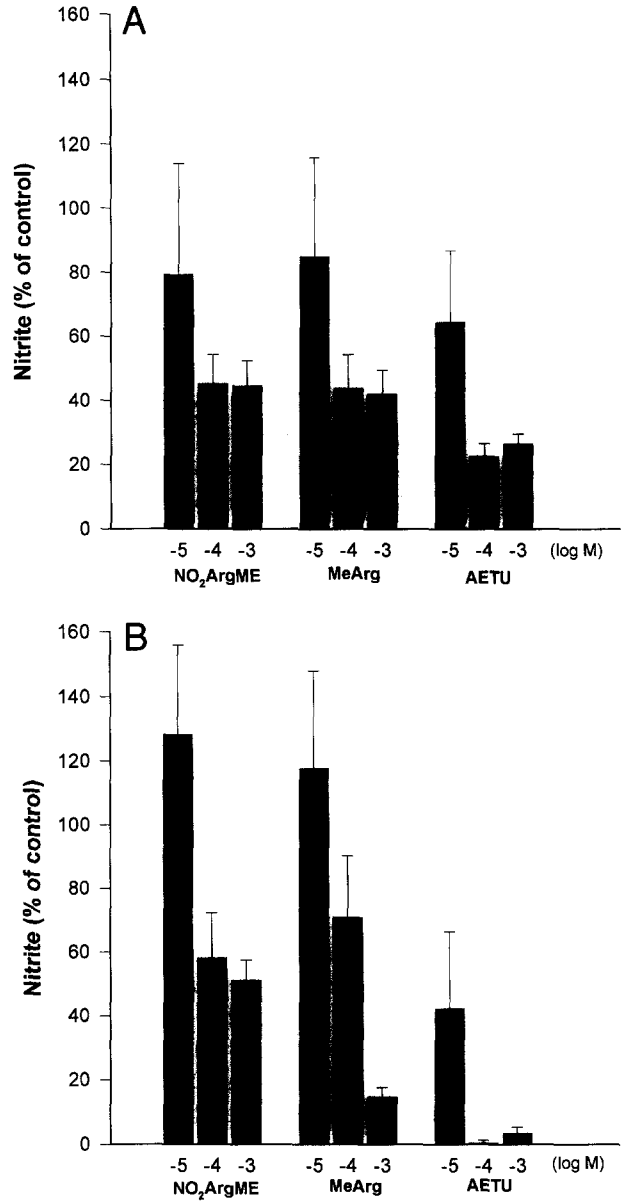

Fig. 2. Effect of N^{ω}-nitro-L-arginine methyl ester (NO_2ArgME), N^{G}-monomethyl-L-arginine monoacetate (MeArg), and *S*-(aminoethyl) isothiourea (AETU) on the increase in nitrite concentration in the media of cultured human osteoarthritic cartilage. Cartilage was stimulated with lipopolysaccharide, LPS (a) or tumor necrosis factor-α, TNF-α (b) for 48 h. Expressed as means ± S.E.M. of stimulated control. Mean value for control group in (a) 118 nmol nitrite/g wet weight/24 h, for (b) 80 nmol nitrite/g wet weight/24 h.

S-(aminoethyl) isothiourea > *S*-methyl isothiourea > aminoguanidine > N^{G}-monomethyl-L-arginine > N^{ω}-nitro-L-arginine = N^{ω}-nitro-L-arginine methyl ester. The E_{50} values for the individual inhibitors were slightly higher in this study than the ones previously reported (Southan et al., 1995), perhaps due to the fact that the cells were stimulated with a mixture of lipopolysaccharide (10 μg/ml) and interferon-γ (50 U/ml), and produced larger amounts of nitrite over 24 h than in the previous study, when lipopolysaccharide alone (1 μg/ml) was used for the stimulus of induction.

The rank order of potency for endothelial NO· synthase, however, was different from the rank order seen for inducible NO· synthase, being N^{ω}-nitro-L-arginine methyl ester = N^{ω}-nitro-L-arginine > N^{G}-monomethyl-L-arginine = *S*-methyl isothiourea > *S*-(aminoethyl) isothiourea > aminoguanidine.

4. Discussion

We evaluated the ability of two members of a novel class of nitric oxide synthase inhibitors, the *S*-substituted isothioureas, to inhibit inducible NO· synthase in bovine chondrocytes and human osteoarthritic cartilage. Both *S*-(aminoethyl) isothiourea and *S*-methyl isothiourea were 4–100 times more potent than N^{G}-monomethyl-L-arginine monoacetate, 10–35 more potent that aminoguanidine, 30–100 times more potent than N^{ω}-nitro-L-arginine and 35–100 times more potent than N^{ω}-nitro-L-arginine methyl ester.

The ability of these NO· synthase inhibitors to inhibit inducible NO· synthase in cultured chondrocytes and cartilage was similar to that reported for cultured murine macrophages (Table 2), as was the rank order of potency (*S*-(aminoethyl) isothiourea > *S*-methyl isothiourea > N^{G}-monomethyl-L-arginine > aminoguanidine > N^{ω}-nitro-L-arginine = N^{ω}-nitro-L-arginine methyl ester). The isothioureas, *S*-(aminoethyl) isothiourea and *S*-methyl isothiourea, ability to inhibit inducible NO· synthase in cartilage and in macrophages was in contrast to their relative ability to inhibit endothelial NO· synthase in bovine endothelial cell homogenates, where the order of potency was N^{ω}-nitro-L-arginine methyl ester = N^{ω}-nitro-L-arginine > N^{G}-monomethyl-L-arginine > *S*-methyl isothiourea = *S*-(aminoethyl) isothiourea > aminoguanidine (Table 2).

The inhibition of inducible NO· synthase activity in chondrocytes afforded by the isothioureas was reduced by excess L-arginine, suggesting a competitive nature of inhibition as demonstrated by Garvey et al. (1994) and Southan et al. (1995). In the latter study, up to 30 mM L-arginine was needed to reverse the effects of the inhibitors in macrophage inducible NO· synthase, which may explain the modest inhibitory effects when the L-arginine concentration in the medium was raised from 0.4 to 1.8 mM (Table 1).

The stimulatory effects of tumor necrosis factor-α and lipopolysaccharide varied from experiment to experiment, reflected in the mean amount of nitrite accumulation, yet the ability of the NO· synthase inhibitors to inhibit inducible NO· synthase in chondrocytes and cartilage was for the most part similar, with *S*-(aminoethyl) isothiourea consistently being the most potent inhibitor of inducible NO· synthase. Interestingly, when *S*-(aminoethyl) isothiourea was evaluated in human osteoarthritic cartilage, more nitrite was detected in lipopolysaccharide-induced medium than in tumor necrosis factor-α-induced medium. Factors such as substrate cellular uptake and means of NO· synthase induction may influence the effectiveness of NO· synthase inhibitors in different cell types. N^{G}-Monomethyl-L-arginine inhibits the uptake of L-arginine in cultured endothelial cells (Bogle et al., 1992). In addition, lipopolysaccharide or tumor necrosis factor-α and interleukin-1 have been shown to enhance uptake of N^{G}-monomethyl-L-arginine in J774 macrophages and cultured endothelial cells (Bogle et al., 1995), competing with L-arginine for the carrier-mediated substrate transporter, whereas N^{ω}-nitro-L-arginine had no effect on uptake. Differences in the cellular uptake of the various *S*-substituted isothioureas (by basal or cytokine-induced transport systems) may have contributed to the difference in potencies that we observed in the various experimental systems (cultured cells versus cartilage; lipopolysaccharide versus tumor necrosis factor-α).

One clinically relevant indicator of the inhibitory potency for inducible NO· synthase inhibitors in endothelial cells is the magnitude of a pressor response elicited by the particular inhibitor. It is noteworthy that *S*-(aminoethyl) isothiourea elicits only modest pressor effects in anesthetized rats (Southan et al., 1995), further suggesting its potential usefulness as an inducible NO· synthase inhibitor in vivo. On the other hand, *S*-ethyl isothiourea, an inducible NO· synthase inhibitor – based on enzyme studies (Garvey et al., 1994; Nakane et al., 1995) – is an extremely potent pressor agent in anesthetized rats (Southan et al., 1995), which makes its in vivo utility questionable.

In conclusion, in the present study, we have observed that *S*-(aminoethyl) isothiourea and *S*-methyl isothiourea, when compared with *N*-substituted L-arginine analogues, are relatively selective inhibitors of inducible NO· synthase in both cultured bovine chondrocytes and human osteoarthritic cartilage. In view of the accumulating evidence that chondrocyte inducible NO· synthase is important in the degradative cascade of arthritis, such inhibitors may have a beneficial role in the management of these debilitating conditions.

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