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Inhibition of nitric oxide synthesis in vascular smooth muscle by retinoids

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1 These studies examine the effect of retinoids on interleukin 1β (IL-1β)-induced nitric oxide synthase (NOS) activity in cultured rat aortic vascular smooth muscle (VSM) cells and isolated rat aortic rings.

2 All-*trans*-retinoic acid (all-*trans*-RA, 0.1–10 μM) and its active analogues produced concentration-dependent inhibition of IL-1β (0.1–10 ng ml^{-1})-induced nitrite production in cultured VSM cells. In contrast, the inactive retinoid, Ro 14-6113 (0.1–10 μM), had no effect on IL-1β-induced nitrite production.

3 Since some of the actions of retinoids are mediated by induction of transforming growth factor beta (TGF-β), its effect on inducible NOS activity in VSM cells was examined. TGF-β produced concentration-dependent (0.1–10 ng ml^{-1}) inhibition of IL-1β-induced nitrite production and the maximum effect (approximately 90% inhibition) was significantly greater than that seen with all-*trans*-RA (approximately 70% with 10 μM). However, an anti-TGF-β antibody (50 μg ml^{-1}) which blocked the effect of exogenous TGF-β (5 ng ml^{-1}) did not significantly reverse the inhibitory action of all-*trans*-RA (10 μM).

4 In addition to inhibiting IL-1β-induced nitrite production, all-*trans*-RA (10 μM) reduced substantially inducible NOS mRNA and protein levels in IL-1β-induced VSM cells ($P<0.01$).

5 Incubation of isolated rat aortic rings with IL-1 β (10 ng ml^{-1}) caused a progressive resistance of the rings to the vasoconstrictor action of phenylephrine (10 nM to 10 μM). This effect was abolished by the addition of the nitric oxide synthase inhibitor L-N^G-monomethyl-L-arginine (L-NMMA, 1 mM). All-*trans*-RA (10 μM) also markedly and significantly reversed this IL-1β-induced vascular hyporeactivity ($P<0.01$).

6 These data show that all-*trans*-RA and other active retinoids are able to block cytokine-stimulated expression of inducible NOS in cultured VSM cells and isolated aortic rings.

Keywords: Retinoic acid; vascular smooth muscle; cytokine; nitric oxide synthase

Introduction

Nitric oxide (NO) is a potent vasodilator generated enzymatically from the guanidino nitrogen group of L-arginine by the action of nitric oxide synthase (NOS) (Moncada *et al.*, 1991). One isoform of NOS is calcium dependent, expressed constitutively in endothelium and produces picomolar amounts of NO (Rees *et al.*, 1989). A second isoform, whose synthesis is induced in endothelium and vascular smooth muscle (VSM) cells by endotoxin and some cytokines, is calcium independent and capable of generating substantially larger (nanomolar) amounts of NO (Radomski *et al.*, 1990). Stimulation of the synthesis of this inducible NOS (iNOS) isoform by endotoxin/cytokines is thought to play a key role in the hypotension of septic shock (Moncada *et al.*, 1991). The profound hypotension that often accompanies the use of cytokines in anti-tumour therapy is probably mediated by the same mechanism (Ochoa *et al.*, 1992).

Glucocorticoids have been shown to block the induction of iNOS in endothelial (Radomski *et al.*, 1990) and VSM (Hirokawa *et al.*, 1994) cells, but these agents are not clinically effective in patients with septic shock (Slotman *et al.*, 1993). Several other inhibitors of iNOS synthesis have been reported, including interleukin 10 (Cunha *et al.*, 1992), nicotinamide (Cetkovic-Cvrlje *et al.*, 1993), cyclosporin A (Muhl *et al.*, 1993), transforming growth factor β (TGF-β; Schini *et al.*, 1992), platelet-derived growth factor (PDGF; Scott-Burden *et al.*, 1992) and thrombin (Schini *et al.*, 1993). However, these compounds are generally of limited therapeutic potential.

The retinoids are a group of natural and synthetic analogues of vitamin A with potent effects on cell growth and differentiation and an important role in embryogenesis (Roberts & Sporn, 1984). In addition, they have been shown to exhibit both anti-inflammatory and immunomodulatory actions (Orfanos & Bauer, 1983), and it is relevant that retinoids can modify cytokine-induced responses (Ishii *et al.*, 1992). Recently, Mehta *et al.* (1994) have reported that all-*trans*-retinoic acid (all-*trans*-RA) blocks the release of tumour necrosis factor (TNF) from peritoneal macrophages stimulated with endotoxin and interferon-γ. Interestingly, they also noted an inhibitory effect on NO production in these cells. We have studied the effect of retinoids on cytokine induction of iNOS in VSM and found that all-*trans*-RA and a number of its analogues are inhibitors of iNOS expression in this tissue.

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Methods

Isolation and primary culture of rat VSM cells

VSM cells were harvested from enzymatically dissociated rat thoracic aorta (male Wistar rats 300–350 g, in-house colony) according to the method of Beasley *et al.* (1991) with some minor modifications (Hirokawa *et al.*, 1994). The cells were suspended in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal calf serum (FCS), L-glutamine 2 mM, penicillin 500 U ml^{-1}, streptomycin 0.5 mg ml^{-1} and amphotericin B 2.5 μg ml^{-1} and seeded into 25 cm^2 flasks. After reaching confluence, cells were passaged by harvesting with trypsin–EDTA, seeded at a ratio of 1:5 into 96-well plates and grown in DMEM supplemented with 10% FCS, L-glutamine 2 mM and antibiotics. The cells were characterized as smooth muscle cells by morphology and immunostaining with a monoclonal antibody to smooth muscle α-actin.

All studies were carried out on VSM cells obtained between the fourth and 20th passage. Cells were stimulated by incubation with interleukin 1β (IL-1β) at different concentrations for varying periods of time up to 24 h. In an earlier study we observed that IL-1β-stimulated nitrite production was reduced in the presence of FCS, which is known to stimulate mitosis (Hirokawa *et al.*, 1994). In order to maximize the nitrite signal and to study quiescent cells, all subsequent experiments were conducted in the absence of FCS. Thus, 24 h prior to study, the medium (100 μl per well) was changed to DMEM without phenol red containing 0.1% bovine serum albumin (BSA) (fatty acid free and low endotoxin) in place of FCS.

Measurement of NO production

NOS activity was assessed by measurement of nitrite production according to the method of Zembowicz & Vane (1992) (Griess reaction) with minor modifications. Supernatants (100 μl) were mixed with 100 μl of the Griess reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthyl-ethylenediamine dihydrochloride in a ratio of 1:1). After a 10 min incubation at room temperature, the absorbance was read at 540 nm using a Titertek Multiscan Plus MKII 96-well plate spectrophotometer. Standard curves were determined using known concentrations of $NaNO_2$. Nitrite levels reflect NOS activity in this system since no other potential source of this ion exists (all media and solutions testing as blank), and NO in aqueous solution containing oxygen is oxidized primarily to nitrite with little or no formation of nitrate (Ignarro *et al.*, 1993).

This assay was used to examine the effect of a variety of agents on NOS activity. Unless indicated all experiments were carried out using IL-1β 10 ng ml^{-1}. Appropriate controls were included in each 96-well plate to overcome variation produced by differences in cell density since VSM cells continue to grow after confluency. All wells in each experiment were judged microscopically to be of similar confluence.

Western blot analysis of iNOS

VSM cells were cultured in 75 cm^2 culture flask with phenol red-free DMEM containing antibiotics, L-glutamine 2 mM and 0.1% BSA for 24 h, and then stimulated with IL-1β 10 ng ml^{-1} in the presence or absence of retinoids or TGF-β for 24 h. At the end of the experiments, culture supernatant was aliquoted and stored for the measurement of nitrite and the cells released using 2 ml of trypsin–EDTA. The cells were washed in phosphate-buffered saline (PBS) (NaCl 8 g l^{-1}, Na_2HPO4 1.15 g l^{-1}; KCl 0.2 g l^{-1}; KH_2PO_4 0.2 g l^{-1}), resuspended in 300 μl lysis buffer (50 mM Tris–HCl, pH 7.4) containing pepstatin A 5 μg ml^{-1}, chymostatin 1 μg ml^{-1}, aprotinin 5 μg ml^{-1}, leupeptin 1 μg ml^{-1}, dithiothreitol 1 mM and phenylmethylsulphonyl fluoride 100 μM, then lysed by freeze and thaw. The lysate was centrifuged at 16,000 *g* for 10 min and the supernatant stored at −70°C until blotted. The protein content of these supernatants were measured with the BCA protein assay reagent (Pierce, IL, USA).

VSM cell protein (12.5–400 μg) was electrophoresed on 7.5% SDS–PAGE, using prestained molecular weight markers as previously described (Hirokawa *et al.*, 1994). Proteins were electroblotted in 20% methanol, Tris 25 mM, glycine 192 mM (pH 8.3) onto nitrocellulose membranes (ECL-Hyperbond, Amersham International, Amersham, U.K.). The membranes were blocked with 4% low-fat milk (Marvel Original, Premier Brands, U.K.) in PBS for 2 h, washed three times in PBS containing 0.05% BSA and 0.05% Tween-20, then incubated with rabbit anti-rat iNOS antiserum (diluted 1:5000 in PBS, 0.1% BSA) (Riveros-Moreno *et al.*, 1993) for 2 h, washed and finally incubated with a 1:20,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (ICN Flow, Oxford, U.K.) for 2 h. The immunocomplexes were visualized using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents, Amersham International) and Hyperfilm (Amersham International, Amersham, U.K.).

Extraction of total RNA from VSM cells and analysis of iNOS mRNA by reverse transcription–polymerase chain reaction (RT–PCR)

The RT–PCR method employed has been validated and described in full in previous publications (Brown *et al.*, 1993; Hirokawa *et al.*, 1994). Briefly, total RNA was extracted from VSM cells cultured in 96-well plates using a modified guanidium isothiocyanate method (Chomczynski & Sacchi, 1987). After ethanol precipitation the RNA was resuspended in 20 μl of nuclease-free water. The amount of mRNA in each RNA sample was quantified by dot-blot hybridization (of 2 μl aliquots) using an oligo-dT 30-mer probe 5'-labelled with [γ^{32}P]-dATP. Aliquots of 4 μl of total RNA were mixed with 25 pmol of a 15-mer random sequence primer, incubated for 2 h at 37°C with 10-U of Moloney murine leukaemia virus reverse transcriptase (Pharmacia, Milton Keynes, U.K.) and the final cDNA samples diluted with nuclease-free water to a volume of 100 μl and stored at 4°C. The primers used for iNOS were as follows: iNOS, sense 5'-CCTACCAAGGTGACCTGAAAG and antisense 5'-TAATGAATTCAATGGCTTGA (Nunokawa *et al.*, 1993). PCR was performed using 2.5 μl of the diluted cDNA sample in a total reaction volume of 25 μl with 1 μM iNOS primers, 1 unit of Taq DNA polymerase (Promega Corporation, Madison, WI, U.S.A.) and 0.25 μCi of [α-^{32}P]-dCTP (>5000 Ci mmol^{-1}; Amersham International). Preliminary experiments were performed to ensure that the PCR was terminated during the exponential phase of amplification (data not shown). The cycling conditions comprised 93°C for 30 s, 60°C for 30 s and 73°C for 1 min. A 15 μl aliquot of each PCR reaction was separated by electrophoresis through a 6% polyacrylamide gel. The gels were exposed to Kodak-X-Omat XAR5 autoradiography film to locate the specific product bands on the gel. These bands were then excised and the amount of incorporated ^{32}P quantified by liquid scintillation counting. The specificity of the iNOS band was verified by DNA sequencing using the dideoxy chain-termination method (Sanger *et al.*, 1977). The relative amounts of template cDNA at the start of the PCR were assessed by measuring the quantity of PCR product during the exponential phase of amplification. For each sample, the counts (in c.p.m.) incorporated into iNOS cDNA were divided by the counts in the oligo-dT dot blot to correct for variation in mRNA extraction.

Studies of iNOS induction in aortic rings

Thoracic arteries were excised from male Wistar rats (300–350 g, in-house colony) and placed in ice-cold Krebs–Henseleit buffer (pH 7.4) containing (mM) $NaHCO_3$ 25, glucose 11, NaCl 118, KCl 4.5, $NaH_2PO_4$1.2, $MgSO_4.7H_2O$ 1.2 and $CaCl_2$1.25) and cleaned of adherent tissue (Parsaee *et al.*, 1992). The arteries were cut into 4 mm rings and de-endothelialized by gentle rotation of the ring around a closed pair of forceps. The rings were transferred to a 96-well plate and covered with 0.2 ml of DMEM containing: L-glutamine 2 mM, penicillin 500 U ml^{-1}, streptomycin 0.5 mg ml^{-1}, 0.1% BSA and, where appropriate, IL-1β and/or all-*trans*-RA at final concentrations of 10 ng ml^{-1} and 10 μM respectively. The plates were incubated for 2 h at 37°C in 95% air and 5% CO_2. After incubation, the rings were suspended in 20 ml organ chambers filled with Krebs–Henseleit buffer (gassed with 95% O_2, 5% CO_2, pH 7.4, at 37°C) by means of two stainless-steel stirrups. The lower stirrup was fixed to the bottom of the chamber and the upper one was connected to a force transducer (Harvard Bioscience, Kent, U.K.) inter-

faced to a MacLab (AD Instruments, Castle Hill, Australia) to record changes in isometric tension. The rings were allowed to relax for 1 h and stretched to a resting tension of 1.5 g. After equilibration, the rings were contracted with phenylephrine (1 μM) several times until steady contraction was observed, and the lack of relaxation with acetylcholine (1 μM) confirmed the absence of endothelium. Contractility of the rings were assessed by tension generated in grams to cumulative concentrations of phenylephrine (10 nM to 10 μM). All experiments were performed in the presence of indomethacin (10 μM).

Materials

Recombinant human IL-1β, recombinant human TGF-β_1 and chicken anti-human TGF-β antibody were obtained from R&D Systems Europe Ltd (Oxford, U.K.) and L-NMMA from Calbiochem (Nottingham, U.K.). 9-*cis*-retinoic acid and RO 14-6113 were generous gifts from Dr Klaus, Hofmann-La Roche (Basle, Switzerland) and all other retinoids were from Sigma (Poole, U.K.). All culture media, antibiotics, antifungal agents and trypsin–EDTA were obtained from ICN Flow (Oxford, U.K.). Plastics for culture were purchased from Falcon (Oxford, U.K.). Fetal calf serum was from Imperial laboratories (West Portway, U.K.). Unless stated otherwise reagents for RT–PCR were from Sigma (Poole, U.K.).

Statistics

Where appropriate, data were analysed by ANOVA (Complete Statistical System, StatSoft software) and statistical significance assessed by unpaired two-tailed Student's *t*-test or variance analysis. Differences were considered statistically significant for $P<0.05$.

Results

Effect of retinoids on iNOS activity in cultured VSM cells

Exposure of cultured VSM cells to IL-1β increases nitrite concentration in the supernatant in both a concentration- and time-dependent manner (Hirokawa *et al.*, 1994). In these studies, mean (±s.e.mean) nitrite concentration in media from unstimulated VSM cells was 2.96 ± 0.40 μM ($n=17$ plates) and from cells cultured for 24 h with IL-1β (10 ng ml^{-1}) 24.6 ± 2.28 μM ($n=17$ plates; $P<0.001$). The IL-1β-induced increase in nitrite formation has been well characterized previously and is inhibited by cyclohexmide and L-NMMA (Hirokawa *et al.*, 1994). The variation between plates was largely due to differences in the density of plated cells; each 96-well plate contained controls appropriate to the treatment examined.

VSM cells were exposed to the following agents during incubation with IL-1β (10 ng ml^{-1} for 24 h: all-*trans*-RA (0.1–10 μM), 13-*cis*-RA (0.1–10 μM), 9-*cis*-RA (0.1–10 μM), all-*trans*-retinal (0.1–10 μM), all-*trans*-retinol (vitamin A, 0.1–10 μM), Ro 14-6113 (an inactive analogue of retinoic acid; 0.1–10 μM) or TGF-β (0.1–10 ng ml^{-1}). All five active retinoids and TGF-β exhibited concentration-dependent inhibition of IL-1β-induced elevation of nitrite levels (Figure 1 and Table 1). Maximum reduction in IL-1β-induced nitrite accumulation by the retinoids (approximately 60–70%) was observed at a concentration of 10 μM; it is noteworthy that TGF-β (10 ng ml^{-1}) produced significantly greater inhibition (approximately 90%) than the maximum effect of the retinoids (Table 1). The inactive analogue, Ro 14-6113, had no effect on IL-1β-induced nitrite production (data not shown).

To investigate the role of TGF-β in mediating the actions of retinoids, we examined the effect of an anti-TGF-β antibody (affinity-purified chicken anti-human TGF-β immunoglobulin; BDA 19, R&D Systems) on the inhibitory action of all-*trans*-RA on IL-1β stimulated cells. At 10 μg ml^{-1} this antibody completely neutralizes the biological activity of 10 ng ml^{-1} rat or human TGF-β assayed by inhibition of proliferation of VSM cells in 10% fetal calf serum (Kirschenlohr *et al.*, 1993; D.J. Grainger, personal communication). Control experiments were performed using

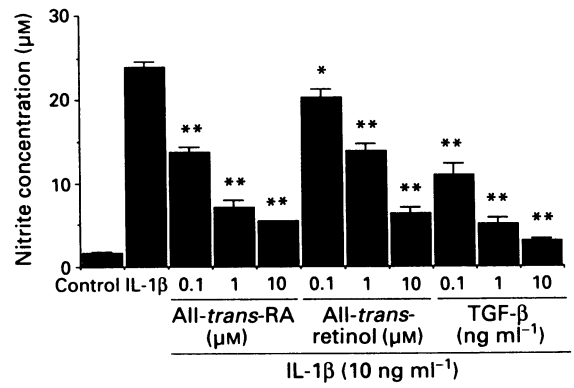

Figure 1 Retinoids and TGF-β inhibit IL-1β-induced NO production by VSM cells in a concentration-dependent manner. VSM cells were cultured with IL-1β (10 ng ml^{-1}) for 24 h in the presence or absence of retinoids or TGF-β. Control represents cells cultured with medium alone. Data are mean ± s.e.mean of four wells and are representative of three independent experiments. *$P<0.05$, **$P<0.001$ compared with control cells incubated with IL-1β alone.

Table 1 Effect of retinoids and TGF-β on IL-1β-induced changes in nitrite concentration

Agent	*Concentration*	*Nitrite concentration* (per cent of control)	n	*Agent*	*Concentration*	*Nitrite concentration* (per cent of control)	n
All-*trans* retinoic acid	0.1 μM	66 ± 3.9	9	All-*trans*-retinol	0.1 μM	90 ± 4.2	3
	1.0 μM	46 ± 4.4	10		1.0 μM	71 ± 10	3
	10	28 ± 2.3	11		10 μM	38 ± 11	3
13-*cis*-Retinoic acid	0.1 μM	72 ± 1.6	3	All-*trans*-retinol	0.1 μM	84 ± 8.9	3
	1.0 μM	52 ± 1.9	3		1.0 μM	72 ± 6.1	3
	10	31 ± 1.8	3		10 μM	43 ± 6.9	3
9-*cis*-retinoic acid	0.1 μM	59 ± 17	3	TGF-β	0.1 ng ml^{-1}	50 ± 6.1	3
	1.0 μM	34 ± 5.1	3		1.0 ng ml^{-1}	23 ± 4.8	4
	10 μM	29 ± 3.6	3		10 ng ml^{-1}	7.2 ± 1.4	6

VSM cells were cultured in 96-well plates for 24 h with IL-1β (10 ng ml^{-1}) ± different concentrations of retinoids (0.1–10 μM) or TGF-β (0.1–10 ng ml^{-1}). Nitrite levels in the supernatant from cells incubated with IL-1β + retinoids/TGF-β are expressed as percentage of levels in supernatant from cells incubated with IL-1β alone (mean ± s.e.mean; n = number of plates).

TGF-β and normal chicken immunoglobulin. In the concentrations employed (up to 50 ng ml^{-1}), anti-TGF-β antibody was able to reduce markedly the inhibitory effect of TGF-β on nitrite production (from approximately 90% to 30%) but the same concentrations had no effect on the action of all-*trans*-RA (Figure 2).

Further insight into the mechanisms underlying the inhibitory action of retinoic acid on IL-1β-induced NO production was provided by measurements of iNOS protein and mRNA levels. Immunoblotting of protein extract from IL-1β-induced cells with anti-iNOS antibody demonstrated a single band of approximately 130 kDa, appropriate for the molecular weight of iNOS (Figure 3). This band was not detected in control cells. Similarly, no band was seen in IL-1β-induced cells co-incubated with TGF-β (10 ng ml^{-1}), while a faint band was detected in IL-1β-induced cells exposed to all-*trans*-RA (10 μM) at the highest protein concentration blotted (400 μg per lane). Consistent with inhibition of iNOS protein synthesis, measurement of iNOS mRNA by RT–PCR showed marked reduction of IL-1β-induced elevation in iNOS mRNA levels (Figure 4).

Effect of retinoic acid on IL-1β-induced relaxation of aortic rings

Preincubation of aortic rings with IL-1β (10 ng ml^{-1}) for 2 h reduced markedly ($P<0.01$) the constrictor response to phenylephrine (Figure 5a). This vascular hyporeactivity was reversed by L-NMMA (1 mM), confirming its dependence on NO generation (Figure 5a). Co-incubation of the rings with all-*trans*-RA (10 μM) and IL-1β inhibited the vasorelaxant effect of the cytokine and restored the vasoconstrictor response to the α-agonist (Figure 5b).

Discussion

These studies demonstrate that all-*trans*-RA inhibits the induction of iNOS by IL-1β in rat VSM cells in culture and in aortic rings. The studies on cultured VSM cells show that this property is common to a number of retinoids and a specific effect of retinoid receptor binding since the inactive retinoid, Ro 14-6113, has no effect in our system. Furthermore, we have shown that the mechanism of action involves a reduction in iNOS mRNA and protein levels. These observations confirm and extend those of Mehta *et al.* (1994) using endotoxin and interferon-γ to induce NO production in mouse peritoneal macrophages.

Retinoids have been shown to influence (activate or inhibit) the expression of a number of genes, including those associated with cell growth and differentiation (Chytil & Haq, 1990), cell matrix turnover (Brinckerhoff *et al.*, 1986; Nicholson *et al.*, 1990; Schule *et al.*, 1991), growth factors such as PDGF (Wang *et al.*, 1990) and TGF-β (Roberts & Sporn, 1992) and co-factors of coagulation (Ishii *et al.*, 1992). They exert their biological effects through at least two distinct classes of intracellular receptors, namely the retinoic acid receptors (RARs), α, β and γ, and the retinoid X receptors (RXR), α, β and γ (Lohnes *et al.*, 1992). RARs in cell extracts exhibit high affinity for both all-*trans*-retinoic acid and 9-*cis*-retinoic acid (in the nanomolar range for both ligands), while RXRs bind 9-*cis*-retinoic acid only at nanomolar concentrations (Heyman *et al.*, 1992; Levin, 1992). These properties, together with the fact that retinoids undergo spontaneous isomerization within cells, has led to the suggestion that 9-*cis*-retinoic acid is the common mediator of the actions of retinoids. Studies of the tissue distribution of retinoic acid receptor mRNA show that RAR-α and RXR-β are expressed in almost all tissues, while RAR-β and RAR-γ and RXRα and RXR-γ display a more restricted pattern of distribution (Mangelsdorf *et al.*, 1992; Wan *et al.*, 1992). There are no data to date on the predominant receptor subtypes expressed in VSM cells.

An interesting observation is that, while the affinity of retinoid receptors for their ligands in cell extracts is in the

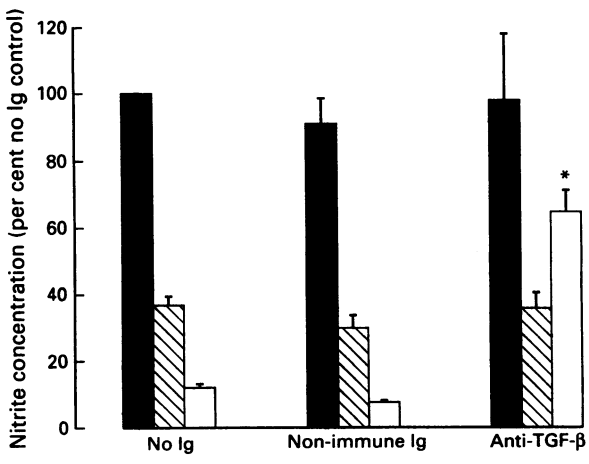

Figure 2 Anti-TGF-β antibody reverses TGF-β inhibition of IL-1β-induced nitrite production, while it fails to reverse the effect of all-*trans*-RA. VSM cells were incubated with IL-1β 10 ng ml^{-1} alone (solid column), IL-1β and all-*trans*-RA 10 μM (hatched column), or IL-1β and TGF-β 5 ng ml^{-1} (open column) in combination with normal chicken immunoglobulin 50 μg ml^{-1} (non-immune Ig) or anti-human-TGF-β chicken polyclonal antibody 50 μg ml^{-1} (anti-TGF-β) for 24 h. No Ig represents VSM cells incubated without immunoglobulin. Nitrite concentration of each well was normalized to mean value of that incubated with IL-1β alone (open column, No Ig). Data are mean ± s.e.mean of three wells and are representative of three independent experiments. *$P<0.05$ compared with cells incubated with IL-1β alone.

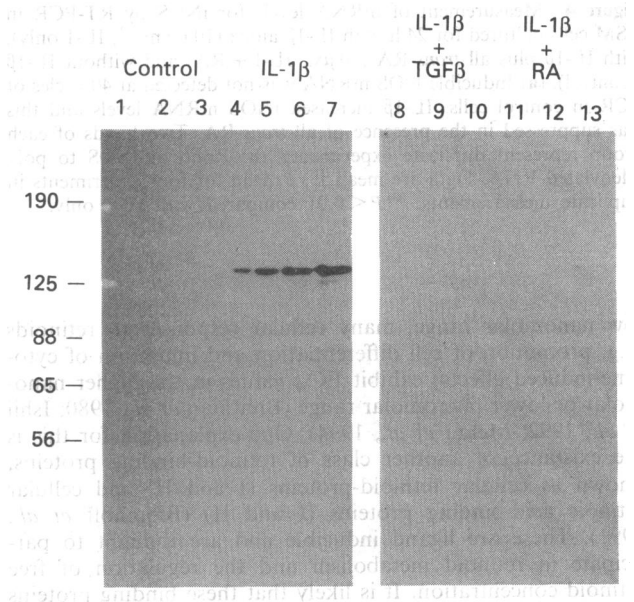

Figure 3 Western blot analysis of iNOS in VSM cells. Supernatant fraction of cell lysate was separated by SDS–PAGE, transferred to nitrocellulose and blotted with anti-iNOS antibody. A single band was seen approximately at 130 kDa, appropriate for the molecular weight of iNOS protein. VSM cells were incubated with medium alone (lanes 1–3) or IL-1β 10 ng ml^{-1} (lanes 4–7), IL-1β plus TGF-β 10 ng ml^{-1} (lanes 8–10) or IL-1β plus all-*trans*-RA (RA) 10 μM (lanes 11–13) for 24 h. Different amounts of protein were subjected to SDS–PAGE (25 μg, lane 4; 50 μg, lane 5; 100 μg, lanes 1,6,8 and 11; 200 μg, lanes 2,7,9 and 12; 400 μg, lanes 3,10 and 13) to enable semi-quantitation of iNOS protein. iNOS band was not detected in cells incubated with IL-1β plus TGF-β and only a faint band was detected in cells incubated with IL-1β plus all-*trans*-RA even at the highest protein concentration examined (400 μg per lane, lanes 10 and 13).

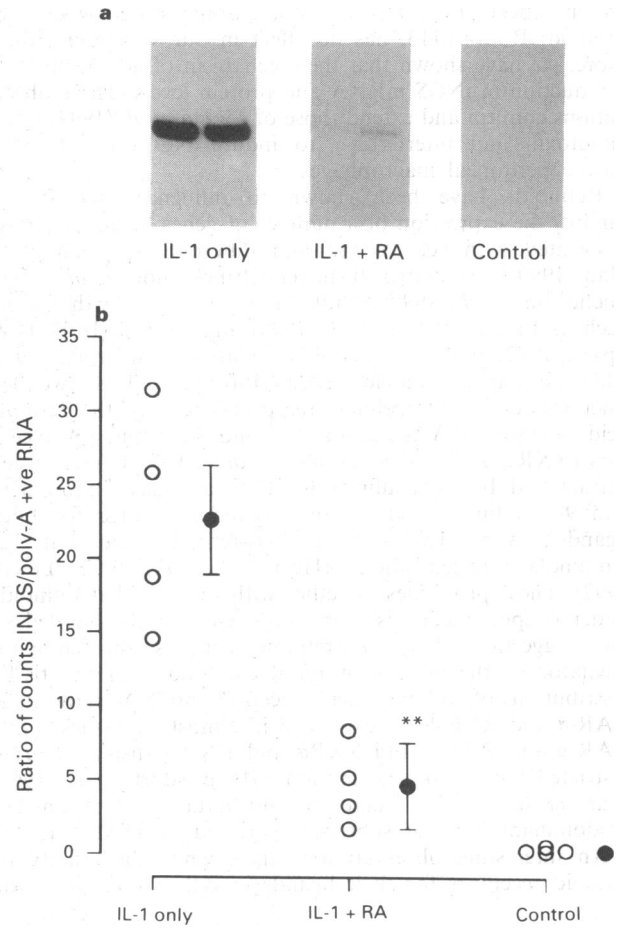

Figure 4 Measurement of mRNA levels for iNOS by RT-PCR in VSM cells cultured for 24 h with IL-1β alone (10 ng ml^{-1}; IL-1 only), with IL-1β plus all-*trans*-RA (10 μM, IL-1 + RA) and without IL-1β (Control). (**a**) Inducible NOS mRNA was not detected at 40 cycles of PCR in control cells. IL-1β increased iNOS mRNA levels and this was suppressed in the presence of all-*trans*-RA. Two bands of each group represent duplicate experiments. (**b**) Ratio of iNOS to poly adenylated RNA. Data are mean ± s.e.mean for four experiments in duplicate measurements. $^{**}P<0.01$ compared with IL-1 only.

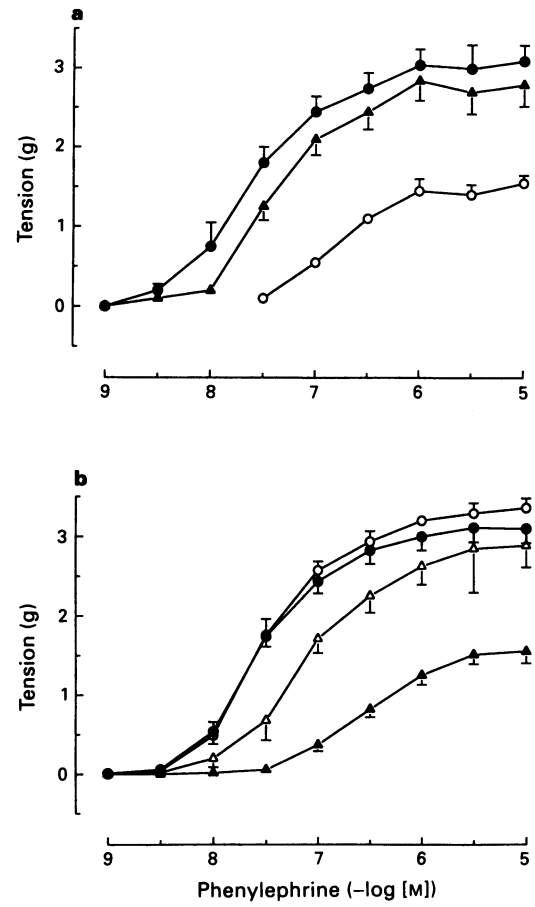

Figure 5 All-*trans*-RA attenuates IL-1β-induced relaxation of aortic rings. Rings, made from isolated rat thoracic aortae, were de-endothelialized and incubated with or without IL-1β (10 ng ml^{-1}) in the presence or absence of RA (10 μM) for 2 h at 37°C. The rings were then suspended in an organ bath and contracted by phenylephrine. (**a**) Rings incubated with IL-1β (○) showed markedly reduced contraction to phenylephrine ($P<0.01$) compared with control rings (●) which was reversed by the NOS inhibitor L-NMMA (1 mM, ▲). (**b**) Contraction of the rings incubated with IL-1β (▲) was again significantly ($P<0.01$) suppressed compared with that of control rings (●), while the rings incubated with all-*trans*-RA alone (○) were not significantly different from control rings. Incubation of rings with IL-1β + all-*trans*-RA (△) significantly restored the response to phenylephrine ($P<0.01$). Data points are mean ± s.e.mean of five rings isolated from 5 different rats.

low nanomolar range, many cellular responses to retinoids (e.g. promotion of cell differentiation and inhibition of cytokine-induced effects) exhibit EC_{50} values in the higher nanomolar or lower micromolar range (Breitman *et al.*, 1980; Ishii *et al.*, 1992; Mehta *et al.*, 1994). One explanation for this is the existance of another class of retinoid-binding proteins, known as cellular retinoid-proteins (I and II) and cellular retinoic acid-binding proteins (I and II) (Blomhoff *et al.*, 1991). These are ligand inducible and are thought to participate in retinoid metabolism and the regulation of free retinoid concentration. It is likely that these binding proteins modify the apparent retinoid concentration–response relationship in intact cells by reducing the free concentration of the ligand.

It is clear that retinoids can regulate gene expression at several different levels. Firstly, it is well established that heterodimers of RAR/RXR (and RXR homodimers) bind to specific DNA sequences known as retinoic response elements (RAREs) on target genes and act as transcription factors. Secondly, RARs, but not RXRs, have been shown to antagonize AP-1 dependent transcription through direct coupling between RAR and AP-1 (Nicholson *et al.*, 1990; Schule *et al.*, 1991). Thirdly, studies on the expression of TGF-β isoforms suggest that retinoids can also act by post-transcriptional mechanisms (Roberts & Sporn, 1992).

The molecular mechanism(s) by which retinoids inhibit the synthesis of iNOS protein remains to be elucidated. Sequence for the promoter region of rat iNOS is not available, but it is noteworthy that a 1749 bp fragment from the 5'-flanking region of the mouse macrophage iNOS gene contains AP-1 binding sites (Xie *et al.*, 1993). An important consideration, however, is that the effects of retinoids on iNOS may be mediated indirectly via its action on other factors. Retinoic acid has, for example, been reported to induce expression of TGF-β (Roberts & Sporn, 1992), which is a potent inhibitor of iNOS expression (data reported here and Schini *et al.*, 1992; 1993). Nevertheless, the effects of all-*trans*-RA in our study were not antagonized by an anti-TGF-β antibody which was able to block the actions of exogenous TGF-β.

In addition to its effects on iNOS expression in this study, RA has also been shown to inhibit cytokine-induced expression of tissue factor and down-regulation of thrombomodulin in endothelial cells (Ishii *et al.*, 1992). This could reduce the thrombotic properties of cytokines, and these properties taken together provide retinoids with a very attractive activity profile as possible therapeutic agents for septic shock.

There is now considerable experience with the use of high doses of all-*trans*-RA in patients with leukaemia (Vahlquist, 1992; Warrell *et al.*, 1993) and its effects on iNOS synthesis and activity could be examined in humans.

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References

BEASLEY, D., SCHWARTZ, J.H. & BRENNER, B.M. (1991). Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrate production in rat vascular smooth muscle cells. *J. Clin. Invest.*, **87**, 602–698.

BLOMHOFF, R., GREEN, M.H., BERG, T. & NORUM, K. (1991). Vitamin A metabolism: new perspectives on absorption, transport and storage. *Physiol. Rev.*, **71**, 952–990.

BREITMAN, T.R., SELONICK, S.E. & COLLINS, S.J. (1980). Induction of differentiation of the human promyelocytic leukemia cell line (HL 60) by retinoic acid. *Proc. Natl. Acad. Sci. USA*, **77**, 2936–2940.

BRINCKERHOFF, C.E., PLUCINSKA, I.M., SHELDON, L.A. & O'CONNOR, G.T. (1986). Half-life of synovial cell collagense mRNA in modulated by phorbol myristate acetate but not all-trans-retinoic acid or dexamethasone. *Biochemistry*, **25**, 6378–6384.

BROWN, L.A., NUNEZ, D.J. & WILKINS, M.R. (1993). Differential regulation of natriuretic peptide receptor messenger RNAs during the development of cardiac hypertrophy in the rat. *J. Clin. Invest.*, **92**, 2702–2712.

CETKOVIC-CVRLJE, M., SANDLER, M. & EIZIRIK, D.L. Nicotinamide and dexamethasone inhibit IL-1 induced nitric oxide production by RINm5F without decreasing messenger ribonucleic acid expression for nitric oxide synthase. *Endocrinology*, **133**, 1739–1744.

CHOMCZYNSKI, P. & SACCHI, A. (1987). Single step method of RNA isolation by acid guanidium isothiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

CHYTIL, F. & HAQ, R. (1990). Vitamin A mediated gene expression. *Crit. Rev. Eucaryotic Gene Expr.*, **1**, 61–73.

CUNHA, F.Q., MONCADA, S. & LIEW, F.Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interleukin-Y in murine macrophages. *Biochem. Biophys. Res. Commun.*, **182**, 1155–1159.

HEYMAN, R.A., MANGELSDORF, D.J., DYCK, J.A., STEIN, R.B., EICHELE, G. EVANS, R.M. & THALLER, C. (1992). 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell*, **68**, 397–406.

HIROKAWA, K., O'SHAUGHNESSY, K., MOORE, K., RAMRAKHA, P. & WILKINS, M.R. (1994). Induction of nitric oxide synthase in cultured vascular smooth muscle cells: The role of cyclic AMP. *B. J. Pharmacol.*, **108**, 786–792.

IGNARRO, L.J., FACADE, J.M., GRISCAVAGE, J.M., ROGERS, N.E. & BYRNS, R.E. (1993). Oxidization of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. USA*, **90**, 8103–8107.

ISHII, H., HORIE, S., KIZAKI, K. & KAZAMA, M. (1992). Retinoic acid counteracts both the downregulation of thrombomodulin and the induction of tissue factor in cultured human endothelial cells exposed to tumour necrosis factor. *Blood*, **80**, 2556–2562.

KIRSCHENLOHR, H.L., METCALFE, J.C., WEISSBERG, P.L. & GRAINGER, D.J. (1993). Adult human aortic smooth muscle cells in culture productive active TGF-β. *Am. J. Physiol.*, **265**, C571–576.

LEVIN, A.A. (1992). 9-Cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRa. *Nature*, **355**, 359–361.

LOHNES, D., DIERICH, A., GHYSELINCK, N., KASTNER, P., LAMPRON, C., LEMEUR, M., LUFKIN, T., MENDELSOHN, C., NAKSHATRI, H. & CHAMBON, P. (1992). Retinoid receptors and binding proteins. *J. Cell Sci.*, **16**, 69–76.

MANGELSDORF, D.J., BORGMEYER, U., HEYMAN, R.A., ZHOU, J.Y., ONG, E.S., ORO, A.E., KAKIZUKA, A. & EVANS, R.M. (1992). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *J. Leukocyte Biol.*, **55**, 336–342.

MEHTA, K., MCQUEEN, T., TUCKER, S., PANDITA, R. & AGGARWAL, B.B. (1994). Inhibition of all-*trans*-retinoic acid of tumour necrosis factor and nitric oxide production by peritoneal macrophages. *J. Leukocyte Biol.*, **55**, 336–342.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MUHL, H., KUNZ, D., ROB, P. & PFEILSCHIFTER, J. (1993). Cyclosporin derivatives inhibit interleukin 1B induction of nitric oxide synthase in renal mesangial cells. *Eur. J. Pharmacol.*, **249**, 95–100.

NICHOLSON, R.C., MADER, S., NAGPAL, S., LEID, M., ROCHETTEEGLY, C. & CHAMBON, P. (1990). Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site. *EMBO J.*, **9**, 4443–4454.

NUNOKAWA, Y., ISHIDA, N. & TANAKA, S. (1993). Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **191**, 89–94.

OCHOA, J.B., CURTI, B., PEITZMAN, A.B., SIMMONS, R.L. BILLIAR, T.R., HOFFMAN, R., RAULT, R., LONGO, D.L., URBA, W.J. & OCHOA, A.C. (1992). Increased circulating nitrogen oxides after human tumor immunotherapy: correlation with toxic hemodynamic changes, *J. Natl. Cancer Inst.*, **84**, 864–867.

ORFANOS, C.E. & BAUER, R. (1983). Evidence for antiflammatory activities for oral and synthetic retinoids: experimental findings and clinical experience. *B. J. Dermatol.*, **S25**, 55–60.

PARSAEE, H., MCEWAN, J.R., JOSEPH, S. & MACDERMOT, J. (1992). Differential sensitivities of the prostacylin and nitric oxide biosynthetic pathways to cytosolic calcium in bovine aortic endothelial cells. *B. J. Pharmacol.*, **107**, 1013–1019.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. USA*, **87**, 10043–10047.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. USA*, **86**, 3375–3378.

RIVEROS-MORENO, V., BEDDELL, C. & MONCADA, S. (1993). Nitric oxide synthase: structural studies using antipeptide antibodies. *Eur. J. Biochem.*, **215**, 801–808.

ROBERTS, A.B. & SPORN, M.B. (1984). Cellular biology and biochemistry of retinoids. In *The Retinoids*, Vol. 2. ed. Sporn, M.B., Roberts, A. & Goodman, D.S., pp. 210–287. New York: Academic Press.

ROBERTS, A.B. & SPORN, M.B. (1992). Mechanistic interrelationships between two superfamilies: The steroid/retinoid receptors and transforming growth factor-B. *Cancer Surv.*, **14**, 205–220.

SANGER, F., NICKLEN, S. & COULSEN, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

SCHINI, V.B., DURANTE, W., ELIZONDO, E., SCOTT-BURDEN, T., JUNQUERO, D.C., SCHAFER, A.I. & VANHOUTTE, P.M. (1992). The induction of nitric oxide synthase activity is inhibited by TGF-B1, PDGFab and PDGFbb in vascular smooth muscle cells. *Eur. J. Pharmacol.*, **216**, 379–383.

SCHINI, V.B., CATOVSKY, S., DURANTE, W., SCOTT-BURDEN, T., SCHAFER, A.I. & VANHOUTTE, P.M. (1993). Thrombin inhibits induction of nitric oxide synthase in vascular smooth muscle cells. *Am. J. Physiol.*, **264**, H611–H616.

SCHULE, R., RANGARAJAN, P., YANG, N., KLIEWER, S., RANSONE, L.J., BOLADO, J., VERMA, I.M. & EVANS, R.M. (1991). Retinoic acid is a negative regulator of AP-1 responsive genes. *Proc. Natl. Acad. Sci. USA*, **88**, 6092–6096.

SCOTT-BURDEN, T., SCHINI, V.B., ELIZONDO, E., JUNQUERO, D.C. & VANHOUTTE, P.M. (1992). Platelet-derived growth factor suppresses and fibroblast growth factor enhances cytokine-induced production of nitric oxide by cultured smooth muscle cells. *Circ. Res.*, **71**, 1088–1100.

SLOTMAN, G.J., FISHER, C.J., BONE, R.C., CLEMMER, T.P. & METZ, C.A. (1993). Detrimental effects of high-dose methylprednisolone sodium succinate on serum concentrations of hepatic and renal function indicators in severe sepsis and septic shock. The methylprednisolone severe sepsis study group. *Crit. Care Med.*, **21**, 191–195.

VAHLQUIST, A. (1992). Long-term safety of retinoid therapy. *J. Am. Acad. Dermatol.*, **27**, S29–S33.

WAN, Y.-J.Y., WANG, L. & WU, T.-C.J. (1992). Detection of retinoic acid receptor mRNA in rat tissues by reverse transcriptase-polymerase chain reaction. *J. Mol. Endocrinol.*, **9**, 291–294.

WANG, C., KELLY, J., BOWDEN-POPE, D.F. & STILES, C.D. (1990). Retinoic acid promotes transcription of the PDGFa-receptor gene. *Mol. Cell. Biol.*, **10,** 6781–6784.

WARRELL, Jr., R.P. DETHE, H., WANG, Z.-Y. & DEGOS, L. (1993). Acute promyelocytic leukemia. *N. Engl. J. Med.*, **329,** 177–189.

XIE, Q.-W., WHISNANT, R. & NATHAN, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon Y and bacterial lipopolysaccharide. *J. Exp. Med.*, **177,** 1779–1784.

ZEMBOWICZ, A. & VANE, J.R. (1992). Induction of nitric oxide synthase activity by toxic shock syndrome toxin 1 in a macrophage-monocyte cell line. *Proc. Natl. Acad. Sci. USA*, **89,** 2051–2055.

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