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Induction of prostanoid, nitric oxide, and cytokine formation in rat bone marrow derived macrophages by activin A

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1 In this study we describe that activin A, a transforming growth factor (TGF) β-like polypeptide affects the expression of inflammatory response genes and their products.

2 In rat bone marrow derived macrophages 15 nM activin A caused the stimulation of prostaglandin (PG) E_2 and thromboxane (TX) A_2 formation, production of nitrite as a marker for nitric oxide (NO) and the release of the cytokines tumour necrosis factor (TNF) α and interleukin (IL) -1β. As shown by mRNA analysis induction of cyclo-oxygenase-2 and inducible nitric oxide synthase by activin A gave rise to the enhanced release of prostanoids and NO.

3 Costimulation of bone marrow derived macrophages with 15 nM activin A and 100 nM 12-O-tetradecanoyl-phorbol 13-acetate (TPA) potentiated the synthesis of prostanoids in a synergistic manner. With respect to NO formation the effect of activin A and TPA was additive.

4 In contrast to the nitrite production activin A induced PGE_2 synthesis was susceptible to tyrosine kinase inhibition by genistein and tyrphostin 46 (IC_{50} was 10 and 20 μM, respectively). This observed inhibition was caused by the selective suppression of activin A induced cyclo-oxygenase-2 mRNA expression. Further, the release of TNFα in the presence of activin A was potentiated by tyrosine kinase inhibition.

5 In summary, we report that activin A exerts proinflammatory activity which results in the formation of prostanoids, NO and cytokines in rat bone marrow derived macrophages. Tyrosine kinase dependent and independent signalling pathways are involved leading to the increased synthesis of these metabolites. Based upon these results, we speculate that activin A may be considered as a possible component of inflammatory processes affecting at least the haematopoietic system.

Keywords: Activin; prostanoid; nitric oxide; macrophage; genistein; cyclo-oxygenase; nitric oxide synthase; cytokine; inflammation

Abbreviations: COX, cyclo-oxygenase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PG, prostaglandin; rmuGM-CSF, recombinant murine granulocyte/macrophage-colony stimulating factor; RT–PCR, reverse transcription polymerase chain reaction; TGF, transforming growth factor; TPA, 12-O-tetradecanoyl-phorbol 13-acetate; TX, thromboxane; TXS, thromboxane synthase

Introduction

Activin A is a homodimeric polypeptide growth factor ($\beta_A\beta_A$) highly homologous to TGF-β (Vale *et al*., 1988) and was originally considered only as gonadal hormone which cause the release of follicle-stimulating hormone in the pituitary (Vale *et al*., 1986). However, the gene encoding activin A is widely expressed in many tissues from embryogenesis through adulthood (Meunier *et al*., 1988). Next to gonadals many different tissues such as liver and kidney are able to synthesize activin A and also bone marrow stromal cells have been shown to release activin A (Yamashita *et al*., 1992). Further, activin A binding has been demonstrated for extragonadal cells, such as leukemic cells (Campen & Vale, 1988), embryonal carcinoma cells (Kondo *et al*., 1989), or fibroblasts (Sakurai *et al*., 1994). Biological responses to activin A are only observed after dimerization of two different receptor types. Both types of activin A receptors are transmembrane serine/threonine kinases which form a heteromeric signalling complex and phosphorylate and activate smad proteins, a class of signal transducers to the nucleus (for review see (Attisano & Wrana, 1998; Massague, 1998)).

Different studies support the function of activin A as a paracrine/autocrine regulator to control multiple physiological processes: control of cell growth (McCarthy & Bicknell, 1993), promotion of neuronal cell survival (Schubert *et al*., 1990), control of hypothalamic oxytocin secretion, and regulation of early embryonic development (Thomson *et al*., 1990). Another important activity is the promotion of erythropoiesis and haematopoiesis, e.g. activin A induces hemoglobin synthesis and megakaryoblastic differentiation and exerts stimulatory action on multipotential hematopoietic precursors (Frigon *et al*., 1992; Yu *et al*., 1987). Furthermore, activin A expression is elevated in inflammatory arthropathies, in interstitial pulmonary fibrosis and in inflammatory bowel disease (Hübner *et al*., 1997; Matsuse *et al*., 1996).

Regarding the influence of activin A on haematopoietic cells and its possible modulatory role in inflammation we were interested in the interaction between activin A, NO, and another family of important mediators, the prostanoid family. Prostanoids are synthesized *via* three enzymatic steps: release of arachidonic acid by phopholipases, conversion of arachidonic acid to prostaglandin endoperoxide by cyclo-oxygenase (COX) and final transformation of the endoperoxide by specific isomerases to the prostaglandins, thromboxane and prostacyclin (Needleman *et al*., 1986). An

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important impact on the formation of prostanoids is given by the expression of the cyclo-oxygenase: two isoforms exist of which COX-1 is constitutively expressed and COX-2 is inducible under inflammatory conditions (for review see (DeWitt *et al*., 1993)).

Similar to COX different isoforms of nitric oxide synthase (NOS) exist. Several cell types and tissues express the constitutive isoform of NOS, an enzyme involved in the maintenance of several aspects of normal cellular physiology, while the induced form (iNOS) is expressed following stimulation with a variety of proinflammatory agents including lipopolysaccharides, cytokines and diverse mitogens (for review see (Moncada *et al*., 1991)). Regarding these aspects, the genes for COX-2 and iNOS enzymes are considered as inflammatory response genes.

Different studies have evoked the interaction of activin A and prostanoids: for amnion cell cultures it has been shown that activin A stimulates the formation of prostaglandins (Petraglia *et al*., 1993) and in murine erythroleukaemia cells activin A induced the activity of thromboxane synthase (TXS) independently of erythroid differentiation (Yamashita *et al*., 1991). Previously, we observed in mouse monocytic cells J774.1 that activin A induces the synthesis of prostanoids *via* induction of COX-1 in dependance of cell differentiation (Nüsing *et al*., 1995). Currently no data are available regarding the interaction of activin A and the NO system.

Cells of the haematopoietic lineage like monocytes and macrophages have large capacities to produce prostanoids (Nüsing & Ullrich, 1990) and NO and are central effector cells in respect to pathological situations such as inflammation or atherosclerosis. In the present study we focused on the modulatory effect of activin A on the synthesis of the inflammatory mediators prostanoids, NO, and the cytokines TNFα and IL-1β.

Methods

Materials

Recombinant human activin A was a kind gift of Y. Eto, Ajinomoto Co. (Kawasaki, Japan) and recombinant murine granulocyte/macrophage-colony stimulating factor (rmuGM-CSF) was kindly provided by F. Seiler, Behring-Werke, (Marburg, Germany). Taq polymerase was purchased from Pharmacia (Freiburg, Germany) and culture media, supplements, and Superscript reverse transcriptase from Gibco BRL (Karlsruhe, Germany). Genistein and tyrphostin 46 were obtained from Biomol (Hamburg, Germany). All other chemicals were of highest grade and purchased from Sigma (Deisenhofen, Germany).

Preparation of rat bone marrow derived macrophages

Bone marrow derived macrophages were prepared as previously described (Barsig *et al*., 1995). Briefly, rat female femures were prepared under sterile conditions and flushed with cell culture media (RPMI 1640), supplemented with 100 μg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, and 10% foetal calf serum. Thereafter, the washed bone marrow derived cells (10^6 cells ml^{-1}) were incubated in culture plates (6-well) in the presence of 10 ng ml^{-1} rmuGM-CSF for 4 days to expand adherent macrophages. Vigorous washings with culture medium removed nonadherent granulocytes and loosely attached dendritic cells. The remaining adherent cells were shown to consist of more than 95% macrophages as identified by nonspecific esterase and F4/80 staining (data not shown). For experiments, culture medium was replaced by fresh medium without fetal calf serum and cells were cultured in the presence of different substances for 20 h as indicated in the legends. In the same incubation culture prostanoids, nitrite and cytokines were determined from the supernatant as described below and RNA was extracted from the adherent cells for further analysis.

Reverse transcription-polymerase chain reaction (RT–PCR) for mRNA detection

Total RNA was extracted from bone marrow derived macrophages using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). After quantification, 1 μg of total RNA was reverse-transcribed into cDNA with sequence-specific primers and PCR was performed as described recently (Nüsing *et al*., 1995; Uhlig *et al*., 1996). The wobble primer 5′-A(G/C)AGCTCAGT(G/T)GA(A/G)CG(C/T)CT-3′ complementary to the 3′-part of COX-1 and -2 was used for reverse transcription of the mRNA of these two

Table 1 Stimulatory action of activin A on the release of different inflammatory metabolites

	Control	*activin A* (15 nM)
PGE_2 (ng ml^{-1})	0.35±0.21	3.28±0.45
TXB_2 (ng ml^{-1})	2.92±0.38	7.62±0.91
NO_2^- (μM)	0.26±0.12	5.88±0.86
TNFα (pg ml^{-1})	34±21	1547±213
IL-1β (pg ml^{-1})	45±18	546±149

Rat bone marrow derived macrophages were prepared and incubated in the absence and presence of 15 mM activin A for 20 h. For measurement of TNFα concentration an aliquot of the supernatant was taken after 4 h. For analysis of the other metabolite samples were collected after 20 h of incubation and stored at −20°C until analysis. Data represent means±s.e.mean of five experiments.

Table 2 Influence of the inhibition of prostanoid formation or cytokine activity on the action of activin

	Nitrite formation	*PGE_2 synthesis*	*TNFα release*	*IL-1β release*
Coincubation of 15 nM activin A with:				
1 μM indomethacin	98±13%	<2%	97±8%	112±15%
Anti-TNFα antibodies	108±11%	75±14%	<1%	105±18%
Anti-IL-1β antibodies	93±14%	95±11%	102±9%	<1%

Rat bone marrow derived macrophages were prepared and incubated with 15 nM activin A alone (control) or in the presence of the idicated antibodies or indomethacin. For determination of TNFα an aliquot of 50 μl were taken after 4 h from the cell supernatant. For IL-1β, nitrite and PGE_2 determination aliquots were collected after 20 h of incubation. The data are given as per cent of control and represent means±s.e.mean of three experiments.

enzymes. For iNOS 5′-AACGTTTCTGGCTCTTGAGCTG-GA-3′, for TXS 5′-GCGTGACACAATCTTGATGTA-GACTCC-3′, and for β-actin the primer 5′-CTAGAAGCATTTGCGGTGGAC-3′ was used. After cDNA synthesis excess primers were removed and PCR amplification was performed using the cDNA template with the following nested primer pairs: for COX-1 the primer pair 5′-ACCCGTCATGTCCAGGGTAA-3′ and 5′-CAGCCCTT-CAATGA(A/G)TACCG-3′, for COX-2 5′-ATCTAGTCTG-GAGTGGGAGG-3′ and 5′-AATGAGTACCGCAAACG-CTT-3′, for iNOS 5′-GCTTCTTCAAGTGGTAGCCA-3′ and 5′-AGTGTCAGTGGCTTCCAGCTC-3′, for TXS 5′-CTAGCTGAAGTGGAACCTGAG-3′ and 5′-TGAGTGC-CAGGAGAGGCTTCT-3′ and for β-actin 5′-CTAGAAG-CATTTGCGGTGGAC-3′ and 5′-CATCACCATTGGC-AATGAGCG-3′ was used. The reactions were cycled 32 times in a cycle profile of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C after a 5 min denaturing step at 95°C. Amplification products were analysed by 2% agarose gel electrophoresis and ethidium bromide staining. No amplification products were found, when the reverse transcription was performed without specific primer or the PCR reaction without template. The identity of the fragments were evaluated by their molecular mass, restriction enzyme analysis or sequencing. Furthermore, samples were assayed in various dilutions to ensure proportionality in the yield of PCR products.

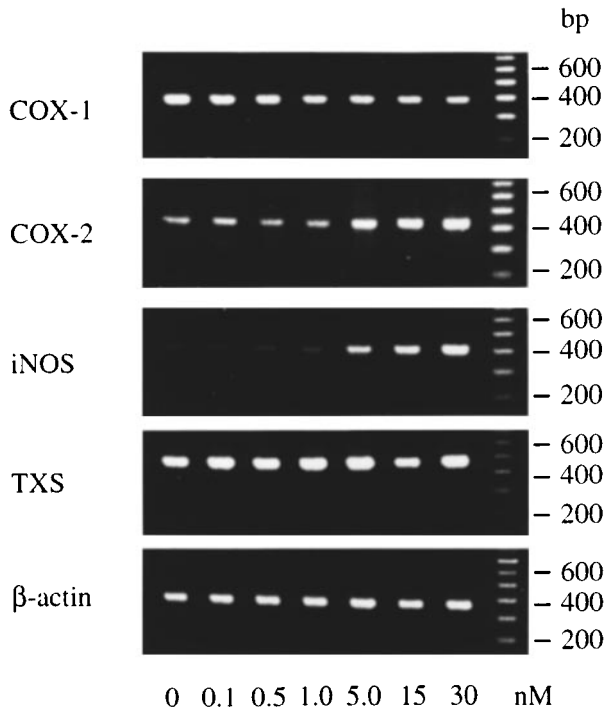

Figure 1 Concentration dependent effect of activin A on expression of mRNA for different proteins of the prostanoid and NO system in rat bone marrow derived macrophages. Cells were incubated for 20 h with the indicated activin A concentrations. Total RNA from adherent cells was isolated and specific mRNA for COX-1, COX-2, TXS, and iNOS was detected by RT–PCR. PCR reactions were performed with primers designed to amplify a 391 base pair (bp) fragment for COX-1, a 420 bp fragment for COX-2, a 406 bp fragment for iNOS or a 450 bp fragment for TXS. Amplification products were analysed by agarose gel electrophoresis and ethidium bromide staining. As a control for RNA levels, amplification of a fragment for β-actin is also shown. Size markers are shown in the right lane.

Table 3 Modulation of the effect of activin A on the production of nitrite and PGE_2

	NO_2^- (μM)	PGE_2 (ng ml^{-1})
Control	0.21±0.08	0.41±0.19
15 nM activin A	5.03±0.64	2.85±0.5
100 ng ml^{-1} LPS	13.24±2.21	5.31±1.26
100 nM TPA	3.22±0.39	1.81±0.73
1 μM dexamethasone	0.18±0.04	0.50±0.21
15 nM activin A+100 ng ml^{-1} LPS	11.91±2.34	8.76±1.8
15 nM activin A+100 nM TPA	14.10±3.61	38.71±8.0
15 nM activin A+1 μM dexamethasone	1.13±0.82	0.60±0.21
15 nM activin A+1 μM aphidicolin	4.87±1.03	2.76±0.39

Rat bone marrow derived macrophages were prepared and incubated with the indicated substances or vehicle for 20 h. Dexamethasone and aphidicolin were given 30 min prior to activin A stimulation. Cell supernatants were collected after 20 h for determination of nitrite and PGE_2. Data represents mean±s.e.mean of 3–5 experiments.

Determination of the prostanoids PGE_2 and TXB_2

After incubation samples were prepared for prostanoid determination as described recently (Schweer *et al*., 1994) with minor modifications. Briefly, sample extracts were spiked with about 10 ng deuterated internal standards and solvent was removed. The methoxime was obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted and the pentafluorobenzylesters were formed. Samples were purified by thin layer chromatography and a broad zone with R_v 0.03–0.39 was eluted. After withdrawl of the organic layers trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to gas chromatography tandem mass spectrometry analysis exactly as described (Schweer *et al*., 1994). Products gained from the above zone were analysed for PGE_2 and TXB_2.

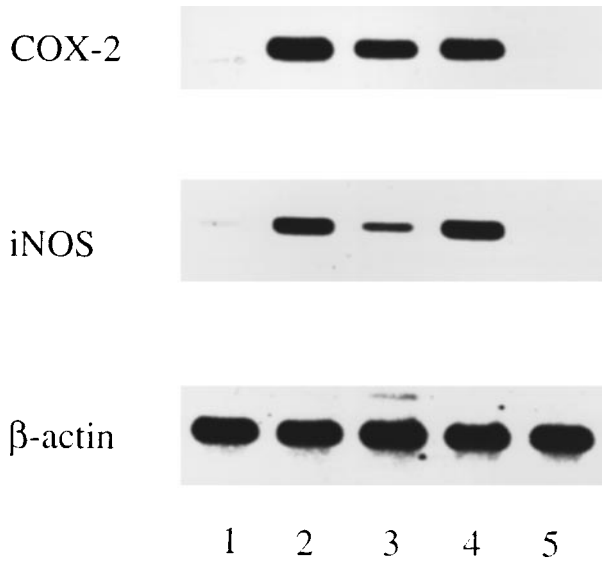

Figure 2 Effects of various agents on the expression of COX-2 and iNOS mRNA. Rat bone marrow derived macrophages were incubated with various agents for 20 h. Dexamethasone was given 30 min prior to activin A stimulation. Total RNA was isolated and expression of COX-2 and iNOS mRNAs was assessed by RT–PCR. Lane 1, control; lane 2, 100 ng ml^{-1} LPS; lane 3, 100 nM TPA; lane 4, 15 nM activin A; lane 5, 15 nM activin A in the presence of 1 μM dexamethasone. β-actin expression was used as internal standard. Experiment represents one of three independent experiments that gave similar results.

Assay of nitrite synthesis

Nitrite production was measured as a marker for NOS activity in the supernatant of cultured cells according to the method of Griess (Green *et al.*, 1982). At the end of the cell incubation, 200 μl of the culture medium was mixed with 1/10th volume of 0.1% naphthylethylenediamine dihydrochloride and 1/10th volume of 1% sulphanilamide in 1.2 M HCl. After incubation for 10 min at room temperature the absorbance at 550 nm was measured and the nitrite concentration was determined using a calibration curve with sodium nitrite standards.

Measurement of TNFα and IL-1β bioactivity

For TNFα determination 4 h and for IL-1β determination 20 h after cell stimulation aliquots were removed from the supernatants and stored at -80°C until analysis. The concentration of the cytokines were determined by bioassay as described (Barsig *et al.*, 1995) using WEHI 164 cells for TNFα and D10N cells for IL-1β determination.

Results

Incubation of cells derived from rat bone marrow with different concentrations of activin A had no effect on cell survival. As in control cells after 4 days the viability of bone

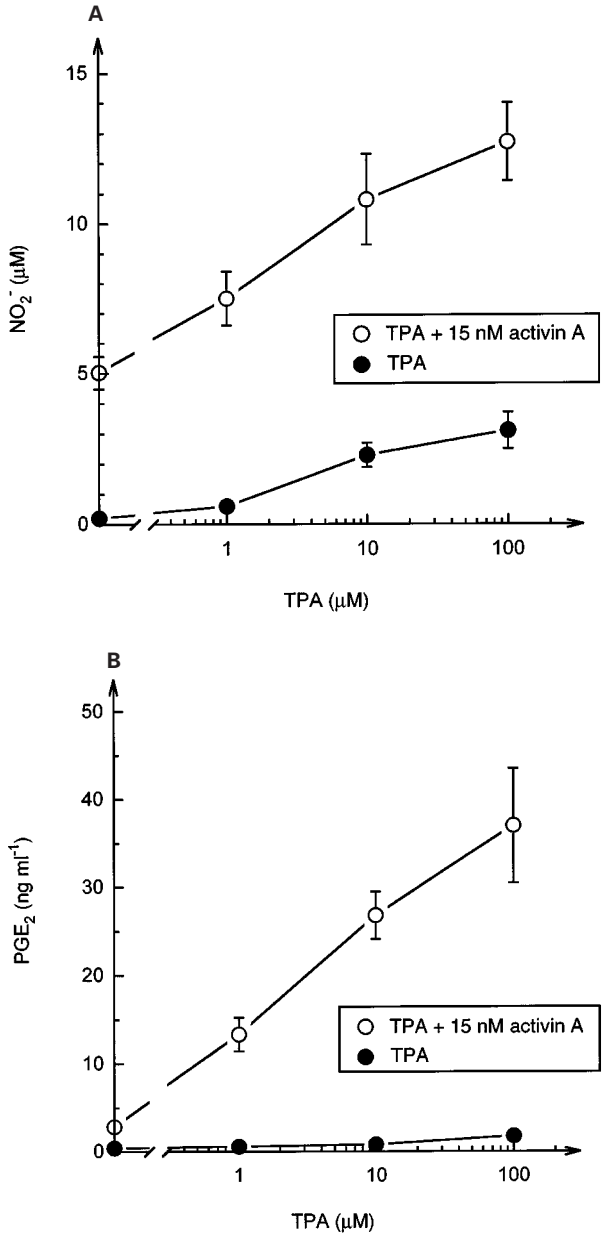

Figure 3 Influence of TPA on the stimulatory action of activin A on nitrite (A) and PGE_2 (B) formation. Rat bone marrow derived macrophages were prepared and incubated for 20 h in the presence of the indicated TPA concentrations alone or in combination with 15 nM activin A. Thereafter supernatants were collected for nitrite (A) and PGE_2 (B) determination. Mean values ± s.e.mean. of four experiments are shown.

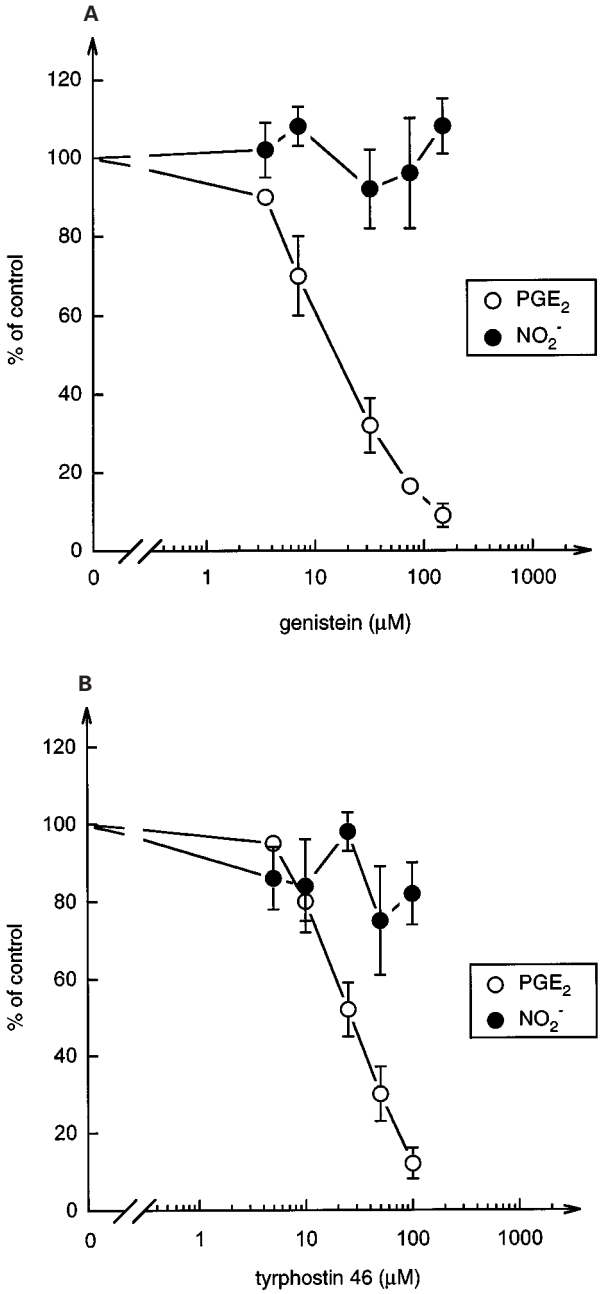

Figure 4 Concentration-dependent effects of genistein (A) and tyrphostin 46 (B) on the activin A induced nitrite and PGE_2 production in rat bone marrow derived macrophages. Cells were pretreated for 30 min in presence of vehicle (control) or the indicated concentrations of the tyrosine kinase inhibitors genistein or tyrphostin 46. 15 nM activin A was added and cells were incubated for 20 h. Supernatants were removed and both PGE_2 and nitrite were determined. Data are shown as per cent of control cells and represent means ± s.e.mean. of three independent experiments.

marrow cells decreased (data not shown). Most likely another cytokine was necessary to promote precursor cells towards a specific differentiation stage. Therefore we used the known differentiation factor GM-CSF to promote macrophage differentiation of bone marrow cells. After incubation with 10 ng ml^{-1} rmuGM-CSF for 4 days more than 95% of the adherent cells exhibited markers typical for macrophages.

Without any stimulation bone marrow-derived macrophages produced only minimal amounts of prostanoids such as PGE_2 and TXB_2, nitrite as a marker for NO formation and cytokines (Table 1). Incubation of these cells with activin A resulted in a time and concentration-dependent increase in nitrite production. The EC_{50} was 6 nM and the dose required for maximal response was about 20–30 nM. Time-course analysis showed that the peak was obtained at about 20 h of incubation (data not shown). In the following experiments we used 15 nM activin A for stimulation. Under this condition, the formation of prostanoids was also increased; PGE_2 was elevated 10 fold and TXB_2 3 fold (Table 1). Furthermore, large amounts of the cytokines TNFα and IL-1β were measurable in the supernatants of these cells. Compared to control TNFα and IL-1β release was induced by activin A 45 fold and 12 fold, respectively (Table 1).

To assess whether activin A induced cytokines act in an autocrine manner on the formation of nitrite and PGE_2 or *vice versa*, we analysed the synthesis of these products in the presence of cytokine antibodies or indomethacin, an inhibitor of prostaglandin synthesis (Table 2). Coincubation with an antibody neutralizing IL-1β activity had no effect on the synthesis of nitrite or PGE_2. IL-1β released during stimulation into the supernatants could no longer be detected by bioassay which confirms the neutralizing activity of the antibody. Use of an antibody directed towards TNFα caused a weak inhibitory effect on the formation of PGE_2 but was without effect on nitrite formation. On the other hand, inhibition of both isoforms of COX by 1 μM indomethacin, did not change the formation of TNFα or IL-1β.

To investigate the molecular basis for the increase in the synthesis of these inflammatory metabolites we used RT–PCR to analyse the mRNA expression of different enzymes involved in prostanoid and NO synthesis. In this experiment rat bone marrow derived macrophages were cultured for 20 h with or without activin A. Total RNA was extracted and used for RT–PCR. Results indicate that the expected fragments are produced (Figure 1). Control cells do not appear to contain mRNA for COX-2 and the message for iNOS could hardly be detected. Dependent on the concentration of activin A a clear upregulation of the transcription for COX-2 and iNOS mRNA was observed. The half maximal effective dose was about 5 nM of activin A. This finding correlates well with the data observed on formation of PGE_2 and nitrite. During the time course expression of COX-1 and TXS mRNA remained unchanged, comparable to the expression of β-actin mRNA which was used as an internal control (Figure 1).

To obtain further insight in the stimulatory potency of activin A we compared the activin A effect with known strong inducers of NO and prostanoid synthesis, i.e. lipopolysaccharide (LPS) and TPA. Although the addition of LPS or TPA for 20 h resulted in a strong increase in nitrite and PGE_2 formation in rat bone marrow derived macrophages, the maximal achievable extent of stimulation was quite different (Table 3). Whereas 100 nM TPA caused a similar increase in product synthesis as 15 nM activin A, the presence of 100 ng ml^{-1} LPS evoked a much stronger formation of nitrite and PGE_2. Interestingly, activin A caused no further increase in LPS action regarding nitrite and prostanoid production in these cells. Dexamethasone at a concentration of 1 μM, known to suppress the expression of inflammatory genes, was capable of blocking both the nitrite formation and the synthesis of PGE_2 induced by activin A. Inhibition of DNA polymerase by 1 μM aphidicolin, thereby blocking cell-cycle dependent cell differentiation (Murate *et al.*, 1991), had no effect on the stimulatory action of activin A. This points towards cell cycle-independent mechanisms triggered by activin A.

In accordance with these results the addition of 100 nM TPA and 100 ng ml^{-1} LPS alone stimulated the expression of

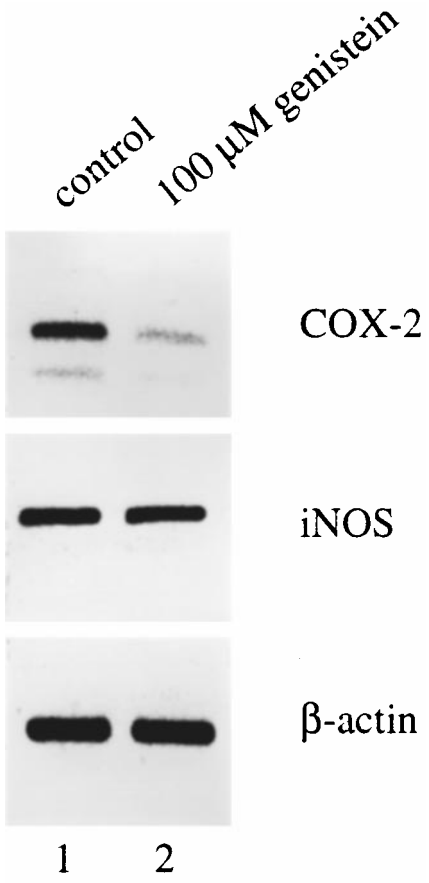

Figure 5 Effect of genistein on the mRNA expression of COX-2 and iNOS in bone marrow derived macrophages. Cells were pretreated for 30 min without (control) or with 100 μM genistein and incubated in the presence of 15 nM activin A for 20 h. Total RNA was isolated and levels of COX-2 and iNOS mRNAs were assessed by RT–PCR. Experiment represents one of three independent experiments that gave similar results. As an internal control β-actin was used.

Table 4 The release of TNFα from rat bone marrow derived macrophages

	TNFα (pg ml^{-1})
Control	24±18
15 nM activin A	1400±185
100 μM genistein	117±34
100 μM tyrphostin 46	182±54
15 nM activin A+100 μM genistein	8288±2413
15 nM activin A+100 μM tyrphostin 46	7208±1958

Rat bone marrow derived macrophages were prepared and stimulated with the indicated substances or vehicle. Genistein and tyrphostin 46 were given 30 min prior to the activin A stimulation. Following 4 h of incubation an aliquot of the supernatant was taken for determination of TNFα formation by bioassay. Mean±s.e.mean of four experiments are shown.

COX-2 and iNOS transcripts and 1 μM dexamethasone totally blocked the activin A induced expression of COX-2 and iNOS message as assessed by RT–PCR (Figure 2).

Next we questioned whether TPA functions as a modulator of activin A action. We observed, that the effect of TPA on nitrite formation at various concentrations was additive with this of 15 nM activin A, suggesting that TPA acts independent of activin A. On the other hand PGE_2 synthesis was stimulated in a synergistic manner by the TPA concentrations used (Figure 3A and B). These experiments depicts the involvement of different induction mechanisms for prostanoid and NO formation.

To examine the transduction mechanisms involved in COX-2 and iNOS induction and TNFα release by activin A and, in particular, the role of tyrosine kinase, we studied the effect of two different tyrosine kinase inhibitors: genistein and tyrphostin 46. Incubation with either compound for 20 h diminished PGE_2 production measured after stimulation with 15 nM activin A (Figure 4A and B). The IC_{50} for tyrphostin 46 was 20 μM and for genistein 10 μM. However, both inhibitors did not cause any change in the synthesis of nitrite. This observation was substantiated by the analysis of mRNA expression for the enzymes COX-2 and iNOS (Figure 5). In agreement to the product formation genistein attenuated selectively the expression of COX-2 mRNA augmented by 15 nM activin A. No effect was observed regarding mRNA expression of iNOS or β-actin.

With respect to the release of TNFα from rat bone marrow-derived macrophages we observed that the tyrosine kinase inhibitors genistein and tyrphostin 46 did not inhibit, but rather potentiated the activin A effect (Table 4). Incubation of the cells with 100 μM genistein or 100 μM tyrphostin 46 in the presence of 15 nM activin A resulted in a 5.9 fold and 5.1 fold increase, respectively, in activin A induced TNFα formation. In the absence of activin A both inhibitors showed only marginal stimulation of TNFα formation.

Discussion

Activin A is broadly distributed and regulates multiple functions in various biological systems, e.g. haematopoiesis. We have shown previously that activin A evokes the upregulation of COX-1 mRNA in mouse monocytic cell line J774.1 which, when untreated, possesses only marginal capacity to synthesize prostanoids (Nüsing *et al*., 1995). We speculate that activin A is responsible for the early onset of developmental processes in these cells leading to the capability to form prostanoids. This finding is in good agreement with the physiological function of monocytes/macrophages.

In the present study we examined the effects of activin A in rat bone marrow derived macrophages. Treatment of bone marrow cells with activin A alone resulted neither in cell differentiation nor in NO or prostanoid release. However, pretreatment with rmuGM-CSF caused the differentiation of the bone marrow cells to macrophage-type cells and rendered them susceptible towards activin A. In contrast to mouse monocytes (Nüsing *et al*., 1995), treatment of rat bone marrow-derived macrophages with activin A gave rise to an inflammatory response demonstrated by a dramatic increase in prostanoid, NO, and cytokine formation. These alterations, at least for the prostanoids and for nitric oxide, were caused by the specific onset of COX-2 and iNOS. These two enzymes are thought as inflammatory response genes. Noteworthy, TGF-β1 has been shown to augment also expression of COX-2 in different cells such as rat epithelial cells and mouse fibroblasts (Gilbert *et al*., 1994a,b).

Various cytokines are known to induce the expression of COX-2 and iNOS in different cell systems. However, in our study the release of TNFα which appeared as early as 4 h after stimulation was not responsible for the increase in synthesis of COX or NOS products. The coincubation of activin A stimulated cells with an anti-TNFα antibody did not alter the responsiveness of rat bone marrow derived macrophages to activin A. Furthermore, coincubation with the COX inhibitor indomethacin had no influence on the release of the cytokines, excluding crosstalk between these mediators.

Further experiments using coincubation of activin A with TPA revealed, that different pathways triggered by activin A lead to the stimulation of NO and PGE_2 synthesis. Phorbol esters such as TPA are strong activators of protein kinase C. With regard to the action of TPA we observed an increase in mRNA expression for COX-2 and iNOS and their enzymatic metabolites, although with different extents. This finding indicates that most likely protein kinase C is involved in the stimulatory pathway. In rat pituitary cells (Katayama & Conn, 1993) observed, that longterm incubation with TPA rendered activin A stimulated release of follicle stimulating hormone more potent, indicating that in these cells protein kinase C is also not required for activin A action but is involved as a modulator of potency of activin A.

A further discriminatory step for activin A on NO and PGE_2 formation is seen by the action of tyrosine kinase inhibitors. Different cell studies have shown that the LPS or cytokine induced elevation of COX-2 and iNOS involves the activity of tyrosine kinase(s). In rat mesangial cells the tyrosine kinase inhibitors genistein and herbimycin A suppressed IL-1β-induced expression of COX-2 and iNOS (Rzymkiewicz *et al*., 1995; Tetsuka & Morrison, 1995) and also in human islets tyrosine kinase inhibitors prevented cytokine-induced expression of COX-2 and iNOS (Corbett *et al*., 1996). The involvement of tyrosine kinase has also been reported for the stimulatory action of LPS using bovine aortic endothelial cells and mouse J774.2 macrophages (Akarasereenont *et al*., 1994). In both cell types erbstatin and genistein dose-dependently blocked COX activity. According to our data only the pathway leading to induction of COX-2 expression can be attenuated by tyrosine kinase inhibitors. Genistein suppressed the activin A induced PGE_2 formation in a concentration dependent manner whereas NO release was unaffected. The IC_{50} value was about 10 μM which is in the range of reported IC_{50} values for genistein in different tyrosine kinase assays. We used a second tyrosine kinase inhibitor, tyrphostin 46 to exclude structure related effects and observed the same dependency. Recently it has been shown that the tyrosine kinase inhibitor radicicol suppresses the expression of COX-2 in LPS stimulated rat alveolar macrophages (Chanmugam *et al*., 1995). In this study p53/56lyn, a member of the src tyrosine kinase family was blocked by radicicol. The identity of the involved tyrosine kinase(s) in our study remains to be elucidated.

The discriminating step between kinase-dependent and -independent enzyme expression might not act at the activin A receptor level, because the activin A receptors belong to the group of receptors with intrinsic serine/threonine kinase activities (Gaddy Kurten *et al*., 1995). Recent studies indicate that activin A type II and type IIB receptor molecules form heterodimeric complexes with two kinds of type I receptor molecules, thereby activating diverse signalling pathways (Attisano *et al*., 1993). The responsiveness of these pathways

to tyrosine kinase inhibitors in rat bone marrow derived macrophages remains elusive.

Recent reports delineated the involvement of activin A in the response to vascular or cutaneous injury and neointimal formation (Hübner *et al*., 1996; Pawlowski *et al*., 1997). In these studies vasoactive agonists such as thrombin and angiotensin II and different growth factors (Hübner & Werner, 1996) were shown to stimulate activin A expression and in turn the mitogenic properties of activin A might support wound repair. Our findings extend this biological range of activin A action to the stimulation of the formation and release of proinflammatory factors such as prostanoids, NO and cytokines. In line with our observation a correlation of activin A expression with the degree of inflammation and the expression of IL-1β was found in the gut of patients suffering from ulcerative colitis and Crohn's disease (Hübner *et al*., 1997). Furthermore, activin A increases migration of inflammatory cells such as monocytes/macrophages and can also be released by these cells (Yu & Dolter, 1997).

In summary, our results indicate that activin A exerts profound inflammatory activity on bone marrow macrophages. Furthermore, in these cells the signalling pathway triggered by activin A leading to increased prostanoid formation is coupled to the action of a genistein- and tyrphostin-sensitive protein tyrosine kinase but not the one for NO formation. It remains to be clarified, if different receptors for activin A are coupled to the different signal transduction pathways or, if the signal splits at some post receptor event into different ways. Our findings strengthen the suggestion that activin A is not only an important modulator of haematopoiesis but may also be a component of specific inflammatory processes.

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