Curcumin suppresses HIF1A synthesis and VEGFA release in pituitary adenomas

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Abstract

Curcumin (diferuloylmethane), a polyphenolic compound derived from the spice plant *Curcuma longa*, displays multiple actions on solid tumours including anti-angiogenic effects. Here we have studied in rodent and human pituitary tumour cells the influence of curcumin on the production of hypoxia inducible factor 1α (HIF1A) and vascular endothelial growth factor A (VEGFA), two key components involved in tumour neovascularisation through angiogenesis. Curcumin dose-dependently inhibited basal VEGFA secretion in corticotroph AtT20 mouse and lactosomatotroph GH3 rat pituitary tumour cells as well as in all human pituitary adenoma cell cultures ($n=32$) studied. Under hypoxia-mimicking conditions ($CoCl_2$ treatment) in AtT20 and GH3 cells as well as in all human pituitary adenoma cell cultures ($n=8$) studied, curcumin strongly suppressed the induction of mRNA synthesis and protein production of HIF1A, the regulated subunit of the hypoxia-induced transcription factor HIF1. Curcumin also blocked hypoxia-induced mRNA synthesis and secretion of VEGFA in GH3 cells and in all human pituitary adenoma cell cultures investigated ($n=18$). Thus, curcumin may inhibit pituitary adenoma progression not only through previously demonstrated anti-proliferative and pro-apoptotic actions but also by its suppressive effects on pituitary tumour neovascularisation.

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Introduction

In addition to substances such as taxol and vincristine, which are already used for chemotherapy of different types of cancer, a growing number of other herbal compounds with putative chemotherapeutic properties have been detected in the past decades (Gupta *et al.* 2010). One of these substances is curcumin, the active ingredient of the spice plant *Curcuma longa*. Curcumin (diferuloylmethane) belongs to the group of polyphenolic herbal compounds and has multiple beneficial effects including anti-tumourigenic actions in different types of human cancers, in particular in tumours of the gastrointestinal tract (Kunnumakkara *et al.* 2008). Although the efficacy of curcumin is currently under investigation in several clinical trials, its usage is limited by its poor bioavailability, which is due to very rapid renal excretion and hepatic degradation (Shehzad *et al.* 2010). More stable curcumin derivatives or curcumin-releasing microparticles, which have successfully been applied in recent studies (Thomas *et al.* 2008, Shahani *et al.* 2010), may overcome this problem in the future.

Tumours of the anterior pituitary represent the second most frequent type of intracranial neoplasms and are mostly benign adenomas derived by monoclonal expansion from transformed hormone- or non-hormone-producing pituitary cells (Asa & Ezzat 2009, Melmed 2011). Clinical symptoms are caused by mass effects of the tumours (headache, visual field loss, etc.) and/or by hyper-secretion of hormones (Asa & Ezzat 2009, Melmed 2011, Perez-Castro *et al.* 2012). Prolactinomas are the most abundant type of pituitary tumours (40–50% of all) followed by endocrine-inactive adenomas (20–25%), somatotropinomas (20%) and cortico-tropinomas (4–8%) (Asa & Ezzat 2009, Melmed 2011). Thyrotropinomas (<1%), gonadotropinomas (<1%) and pituitary carcinomas (<0·1%) are rare entities of pituitary tumours (Saeger *et al.* 2007). Medical treatment regimens with dopamine agonists and somatostatin analogues are established for prolactinomas and somatotropinomas

respectively (Colao & Savastano 2011, Giustina *et al.* 2011), and promising treatment results with temozolomide have been reported in a few cases of pituitary carcinoma (Raverot *et al.* 2010). The other pituitary adenoma types as well as drug-resistant prolactinomas and somatotropinomas have to be removed by transsphenoidal or transcranial surgery. However, if critically localised, the tumours cannot be completely removed and start to re-grow. Thus, alternative options for the pharmacological treatment of pituitary adenomas are needed.

Like any type of solid tumours, expanding pituitary adenomas develop an intratumoural vascular network, which is necessary to transport oxygen and nutrients to the tumour cells and to remove intratumoural waste products (Turner *et al.* 2000, Carmeliet 2003). Tumour neovascularisation is induced by intratumoural hypoxia, which leads to an increase in hypoxia inducible factor 1α (HIF1A), the regulated subunit of the transcription factor HIF1 (Harris 2002, Hickey & Simon 2006). The latter is composed of two subunits, the constitutively expressed HIF1B and HIF1A, which is absent under normoxic conditions but is rapidly up-regulated under hypoxia (Webb *et al.* 2009) and stabilised in pituitary tumour cells by RSUME (Carbia-Nagashima *et al.* 2007, Shan *et al.* 2012). HIF1 regulates multiple genes to induce mechanisms to overcome cellular hypoxia; among them angiogenesis, a complex process in which multiple angiogenic factors induce, in a coordinated manner, the sprouting of new vessels from already existing ones and direct their growth into the expanding tumour (Carmeliet 2003). One of the most important angiogenic factors is vascular endothelial growth factor A (VEGFA), which stimulates vessel growth predominantly through VEGF receptor type 2 (VEGFR2; Ferrara 2004, Carmeliet 2005).

We and others have previously shown that curcumin has anti-proliferative and pro-apoptotic effects in cultured pituitary tumour cells and could inhibit the growth of experimentally induced GH3 cell pituitary tumours in athymic nude mice (Miller *et al.* 2008, Schaaf *et al.* 2009, Bangaru *et al.* 2010). In addition, we have demonstrated that curcumin could suppress VEGFA mRNA synthesis and secretion in a special non-hormone-producing pituitary cell type, the so-called folliculostellate (FS) cells (Schaaf *et al.* 2010). In the present paper, we have studied whether curcumin is a suppressor of both VEGFA secretion and HIF1A production in rodent and human pituitary tumour cells under normoxic and hypoxic conditions.

Materials and Methods

Materials

Cell culture materials and reagents were obtained from Life Technologies, Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), Seromed (Berlin, Germany), Flow Cytometry Standards Corp. (Meckenheim, Germany) and Sigma.

Rodent and human pituitary tumour cells

Corticotroph AtT20 mouse and lactosomatotroph GH3 rat pituitary tumour cell lines were cultured as reported (Shan *et al.* 2012) in DMEM (pH 7·3) supplemented with 10% (v/v) FCS, 2×10^{-3} mol/l glutamine, 0·5 mg/l partricin and 10^5 IU/l penicillin–streptomycin.

Primary human pituitary tumour cell cultures were prepared from the tissue of 22 non-functioning, four somatotrophs, four corticotrophs and two lactotroph adenomas (Table 1) that we received from different neurosurgical centres within 24 h after transsphenoidal surgery. The experiments with human material were performed after approval of the Local Ethics Committee (Ethics grant no. 141-07) and informed written consent was received from each patient whose pituitary adenoma tissue was used in the study. To obtain primary human pituitary tumour cell cultures, the adenoma tissue was enzymatically and mechanically dispersed as previously described (Renner *et al.* 1998) and the tumour cells obtained were carefully washed by repeated centrifugation. The cells were seeded onto wells of 48-well plates at a density of 100 000 cells/well and were cultured in the above-described cell culture medium. Human pituitary adenoma cells were attached within 48 h and were then used for stimulation experiments. Owing to the limited amount of human adenoma tissue, enough cells were not obtained for primary cell cultures from each adenoma to perform all experiments in parallel.

Treatment of cells

For *in vitro* experiments HPLC-purified plant extract of curcumin (90%; Sigma) was used. A stock solution of 10 mM curcumin (prepared in DMSO) was diluted with cell culture medium to obtain final doses of 0·5–30 μM curcumin for cell treatments for various time periods as indicated. Controls with 0·3% (v/v) DMSO corresponding to the amount present in the highest curcumin dosage of 30 μM were prepared in each experiment to exclude unspecific, toxic effects. DMSO alone had no effect on cell viability determined by ethidium bromide/acridine orange staining.

To study the effect of hypoxia on VEGFA and HIF1A production, cells were treated with cobalt chloride ($CoCl_2$), a well-established substance used to mimic hypoxic conditions (Ebert & Bunn 1999, Masson & Ratcliffe 2003, Yuan *et al.* 2003, Webb *et al.* 2009). In a previous study, we have shown that $CoCl_2$ treatment and hypoxia (1% O_2) had similar effects on HIF1A and VEGFA production in pituitary tumour cells (Shan *et al.* 2012). $CoCl_2$ was diluted in cell culture medium and was used at dosages between 31·25 and 250 μM. In experiments in which the effect of curcumin on $CoCl_2$ was studied, curcumin was added to the tumour cell cultures 30 min before $CoCl_2$ application. The viability of the $CoCl_2$-treated pituitary tumour cells was routinely controlled at the end of the experiments with the ethidium bromide/acridine orange method to exclude unspecific toxic effects of $CoCl_2$.

Table 1 Overview of the pituitary adenomas studied and the influence of curcumin on basal and CoCl$_2$-induced VEGFA secretion in primary pituitary tumour cell cultures

Tumour	Sex/age	VEGFA secretion			
		Basal VEGFA[a]	Inhibition by curcumin[b] (%)	CoCl$_2$-induced VEGFA[c]	Inhibition by curcumin after CoCl$_2$ treatment[d] (%)
ST1	F/44	92·6±10·3	44·8[‡]	ND	ND
ST2	F/63	41·5±7·2	56·1[‡]	ND	ND
ST3	F/40	139·8±17·3	45·3[‡]	1158·9±115·3	57·1[§]
ST4	M/37	122·6±28·1	39·9[‡]	989·2±118·9	77·7[§]
NT1	F/69	48·2±2·4	59·4[‡]	ND	ND
NT2	M/57	47·6±5·2	64·7[‡]	ND	ND
NT3	M/58	449·8±35·5	50·6[‡]	ND	ND
NT4	F/77	327·0±39·6	50·1[‡]	ND	ND
NT5	F/40	35·4±2·5	61·5[‡]	220·5±18·1	70·1[§]
NT6	F/24	67·6±8·6	73·1[‡]	231·3±21·4	55·7[§]
NT7	M/42	483·1±40·9	79·1[‡]	ND	ND
NT8	M/52	86·9±12·3	58·3[†]	294·6±46·7	70·4[§]
NT9	M/71	188·2±16·4	28·2[‡]	ND	ND
NT10	M/79	80·7±3·8	57·6[‡]	184·4±23·1	75·9[§]
NT11	F/50	125·2±12·4	77·9[‡]	509·4±83·8	75·7[§]
NT12	M/61	377·2±38·3	87·1[‡]	ND	ND
NT13	M/59	93·2±8·4	72·4[‡]	245·1±12·5	66·0[§]
NT14	F/71	92·1±9·8	56·6[‡]	402·4±35·3	73·6[§]
NT15	M/38	225·4±13·7	34·9[‡]	ND	ND
NT16	M/68	16·7±1·8	65·7[‡]	93·6±9·9	66·6[§]
NT17	M/45	31·1±4·9	56·9[‡]	128·8±5·3	73·5[§]
NT18	F/68	62·2±6·3	75·0[‡]	379·1±41·9	67·8[§]
NT19	M/62	82·8±9·3	67·4[‡]	213·3±14·6	37·2[§]
NT20	M/51	311·6±24·6	65·5[‡]	630·4±68·1	59·9[§]
NT21	M/65	71·6±5·1	45·5[†]	193·1±18·2	54·8[§]
NT22	M/42	124·2±8·1	61·0[‡]	414·6±33·1	64·1[§]
CT1	M/57	22·5±1·3	26·9[*]	ND	ND
CT2	M/56	95·8±11·2	56·2[‡]	ND	ND
CT3	F/33	76·9±8·6	54·5[‡]	ND	ND
CT4	M/37	28·3±2·9	39·6[‡]	317·2±9·7	55·1[§]
LT1	M/71	48·2±3·3	54·1[‡]	ND	ND
LT2	M/31	31·4±3·5	72·3[‡]	142·1±19·5	85·2[§]

VEGFA, vascular endothelial growth factor A; ST, somatotroph tumour; NT, non-functioning tumour; CT, corticotroph tumour, LT, lactotroph tumour; F, female; M, male; ND, not done. [*]$P<0·05$, [†]$P<0·01$, [‡]$P<0·001$ vs basal VEGFA; [§]$P<0·001$ vs CoCl$_2$-induced VEGFA.
[a]Picogram VEGFA per millilitre after 24 h incubation.
[b]Percent reduction of basal (=100%) VEGFA.
[c]Picogram VEGFA per millilitre after treatment with 125 μM CoCl$_2$ for 24 h.
[d]Percent reduction of CoCl$_2$-induced VEGFA (=100%).

Even after the longest treatment periods (72 h) with the highest CoCl$_2$ dose (250 μM), we observed no changes in the viability of the tumour cells.

Real-time RT-PCR

To study the influence of curcumin on the mRNA synthesis of *VEGFA* and *HIF1A* in pituitary tumour cells, real-time RT-PCR was performed as previously described (Shan *et al.* 2012). In brief, RNA was extracted from GH3 and AtT-20 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. One microgram total RNA was reverse transcribed using random hexanucleotides under restrictive conditions (Pagotto *et al.* 2000). Quantitative real-time RT-PCR was performed with cDNA samples of

GH3 and AtT-20 cells as templates. The amplification reactions of 35 cycles were carried out with specific primers for mouse HIF1A (sense: 5′-TAC TGA GTT GAT GGG TTA TGA-3′, antisense: 5′-AAG GCA GCT TGT ATC CTC-3′), mouse VEGFA (sense: 5′-TCT ACC AGC GAA GCT ACT GCC-3′, antisense: 5′-TTA CAC GTC TGC GGA TCT TG-3′), rat HIF1A (sense: 5′-GCA GCG ATG ACA CGG AAA C-3′, antisense: 5′-CAT ATC GCT GTC CAC ATC AAA-3′) and rat VEGF (sense: 5′-GAC GTC TAC CAG CGC AGC TAT-3′, antisense: 5′-AAA TGC TTT CTC CGC TCT GAA-3′). Mouse β-actin (sense: 5′-AGT ATC CAT GAA ATA AGT GGT TAC AGG-3′, antisense: 5′-CAC TTT TAT TGG TCT CAA GTC AGT GTA-3′) and rat HPRT (sense: 5′-ACT GAA AGA CTT GCT CGA GAT-3′, antisense: 5′-CCG TTG ACT GGT

CAT TAC AG-3′) were used as references. Absolute Blue QPCR SYBR Green Mix (Thermo Scientific, Braunschweig, Germany) was used following the manufacturer's instructions. PCR amplifications were performed in a MiniOpticon Real-Time PCR Detection System (Bio-Rad), and the data were analysed using CFX Manager Software for MiniOpticon (version 1.5, Bio-Rad). For each sample, the values were normalised by the amount of β-actin. All experiments were carried out in triplicates.

Measurement of VEGFA

For the determination of VEGFA secretion, 50 000 AtT20 or GH3 cells or 100 000 human pituitary adenoma cells were seeded into 48-well plates containing 0·5 ml culture medium. After attachment, the cells were stimulated as indicated and VEGFA was measured in the cell culture supernatant using mouse-, rat- or human-specific VEGFA ELISA kits (R&D Systems, Wiesbaden, Germany) following the manufacturer's instruction. All secretion experiments were carried out in quadruplicates.

HIF1A analysis by western-immunoblotting

To study HIF1A protein production, AtT20 and GH3 cells were seeded into a six-well plate, grown to about 80% confluence and then treated with curcumin/$CoCl_2$ as indicated. Cells from each well were separately harvested by scraping and applied to protein extraction. Human adenoma cells were cultured in 48-well plates (100 000 cells/well) and cells from eight wells were treated under the same conditions and pooled for protein extraction. Cell lysates were harvested in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma). The protein concentrations of the cell lysates were determined by Bradford dye assay (Bradford 1976). Thirty micrograms of each sample were separated by a precast Tris–glycine gel (Anamed, Darmstadt, Germany) in an electrophoresis apparatus (Invitrogen) and then transferred on a nitrocellulose membrane (Hybond ECL) with Novex Semi-Dry Blotter (Invitrogen). The membranes were then blocked in 5% (w/v) non-fat milk solution (dissolved in TBS/0·1% Tween) and incubated overnight at 4 °C with mouse monoclonal antibody against HIF1A (R&D Systems) diluted 1:500 in 2·5% (w/v) non-fat milk solution. After washing three times with TBS/0·1% (v/v) Tween, the membranes were incubated with HRP-conjugated anti-mouse antibody (Cell Signaling, Frankfurt, Germany) diluted 1:2000 in 2·5% (w/v) non-fat milk solution. The ECL system and hyperfilm (GE Healthcare, Munich, Germany) were used for membrane visualisation.

Statistical analysis

Each of the experiments with rodent pituitary tumour cell lines was repeated at least three times. The individual experiments with cell lines and primary human pituitary adenoma cell cultures were performed using quadruplicate wells. Results are expressed as mean ± s.d. One-way ANOVA was used to compare variables, and $P < 0·05$ was considered as significant. The statistical analyses were performed with SigmaStat 2.0 (SPSS, Inc., Ehningen, Germany).

Results

Effect of curcumin on basal VEGFA production in pituitary tumour cells

Quantitative RT-PCR showed that 24 h of curcumin application led to a dose-dependent reduction of the basal VEGFA mRNA synthesis in both GH3 cells and AtT20 cells (Fig. 1A and B). Consequently, the VEGFA secretion in both pituitary tumour cell lines was dose dependently suppressed by curcumin not only after 24 h but also after longer exposure times (Fig. 1C and D).

In primary cell culture, all 32 human adenomas studied secreted VEGFA under basal conditions. The release of VEGFA per 100 000 adenoma cells per well was highly variable resulting in different concentrations of VEGFA in the cell culture supernatants ranging from 16·7 ± 1·8 to 483·1 ± 40·9 pg/ml VEGFA after 24 h (Table 1). Treatment of primary adenoma cell cultures with different doses of curcumin (1–30 μM) led to a dose-dependent suppression of basal VEGFA production (Fig. 2 shows representative results for different human pituitary adenomas under different treatment regimens). Significant suppression was achieved with 20 and 30 μM curcumin in all adenomas (in Table 1 the reduction of basal VEGFA in percent is summarised for the 24 h treatment with 30 μM curcumin) and with 10 μM in most (83%) tumour cell cultures. However, in some cases even 5 and 1 μM curcumin significantly inhibited basal VEGFA production (Fig. 2). Time course studies of up to 72 h showed that the increase in VEGFA secretion was consistently suppressed by curcumin. The suppressive effect of curcumin was not dependent on the type of adenoma or the basal secretion rate of VEGFA.

DMSO, which is widely used as solvent for lipophilic drugs, has multiple *in vitro* and *in vivo* actions on its own (Santos *et al*. 2003), including stimulatory and inhibitory effects on growth hormone (GH) and prolactin (PRL) production (Nagasawa 1983, Abdel-Haq *et al*. 2000). However, careful searches of the literature gave no hints that DMSO itself had effects on VEGFA or HIF1A production. Nevertheless, the effects of the maximum vehicle dose (0·3% DMSO) on HIF1A and VEGFA production were controlled in all experiments with the exception of a few human adenomas in which very limited tissue material was available. DMSO (0·3%) had no significant effect on basal mRNA synthesis and secretion of VEGFA in AtT20 and GH3 cells (Fig. 1) and in those human adenoma cultures in which the effect of DMSO was tested (not shown).
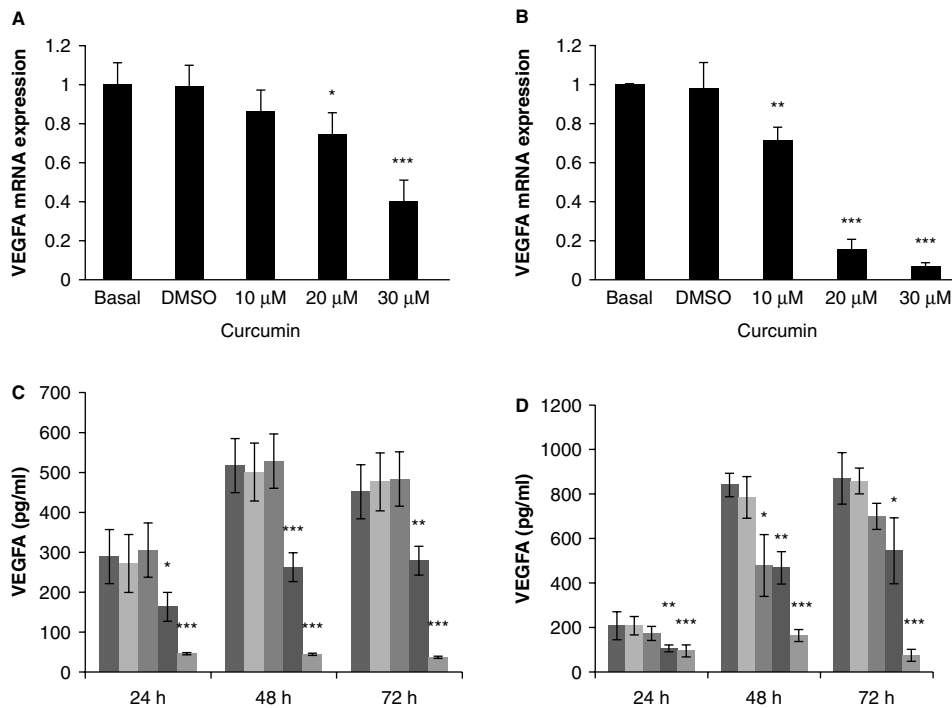
Figure 1 Effect of curcumin on the basal mRNA synthesis and release of VEGFA in endocrine pituitary tumour cell lines. 24 h treatment with curcumin dose-dependently inhibited mRNA synthesis in corticotroph AtT20 (A) and lactosomatotroph GH3 (B) cells, whereas DMSO (0·3% in culture medium) had no effect. Data are expressed as relative mRNA expression in comparison with basal (=1). The secretion of VEGFA by AtT20 (C) and GH3 (D) cells was also dose-dependently suppressed by curcumin but not by DMSO after different treatment periods. For each time point grouped bars correspond – from left to right – to basal, DMSO, and 10, 20 and 30 μM curcumin treatment conditions. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs corresponding basal conditions.

Influence of curcumin on HIF1A production in pituitary tumour cells

We have recently demonstrated that the treatment of pituitary tumour cells with the hypoxia-mimicking agent $CoCl_2$ had a similar effect on HIF1A production as observed under hypoxic (1% O_2) conditions (Shan *et al*. 2012). Therefore, in this paper only the effect of curcumin on $CoCl_2$-induced *HIF1A* mRNA synthesis and protein production was studied in AtT20 and GH3 cells and in eight human pituitary adenomas, from which sufficient cells were available. In all experiments, curcumin was added to the cell cultures 30 min before $CoCl_2$ treatment. Twenty-four hours treatment with curcumin dose dependently suppressed not only $CoCl_2$-induced but also basal *HIF1A* mRNA synthesis in both GH3 (Fig. 3A) and AtT20 cells (not shown). In a previous paper, we have shown that HIF1A protein levels reach maximum levels in pituitary tumour cells after 3 h treatment with $CoCl_2$ (Shan *et al*. 2012). Pretreatment of pituitary tumour cell lines with different dosages of curcumin for 30 min and subsequent application of $CoCl_2$ for 3 h led to a significant and dose-dependent reduction in HIF1A protein formation both in AtT20 and GH3 cells (Fig. 3). The same was observed in eight human adenoma cell cultures, in which curcumin also

suppressed the $CoCl_2$-induced HIF1A protein production (representative results are shown in Fig. 3). DMSO (0·3%) had no effect on HIF1A production in the cell lines and adenoma cell cultures (not shown).

Influence of curcumin on the VEGFA secretion of pituitary tumour cells under hypoxia-mimicking conditions

As we have previously shown that the VEGFA production by AtT20 cells could not be stimulated under hypoxia-mimicking conditions for reasons that are still not known (Shan *et al*. 2012), the effect of curcumin on $CoCl_2$-induced mRNA synthesis and secretion of VEGFA were studied only in the GH3 pituitary tumour cell line. Increasing doses (62·5, 125 and 250 μM) of $CoCl_2$ significantly increased VEGFA secretion, which was dose-dependently suppressed by curcumin (Fig. 4B). The latter also inhibited the $CoCl_2$-induced VEGFA mRNA synthesis, whereby 10 μM curcumin already completely abolished the $CoCl_2$-stimulated VEGFA mRNA production (Fig. 4A).

In 16 human adenoma cell cultures, the effect of hypoxia-mimicking $CoCl_2$ treatment was studied and in all tumour cell cultures a strong increase in VEGFA production was
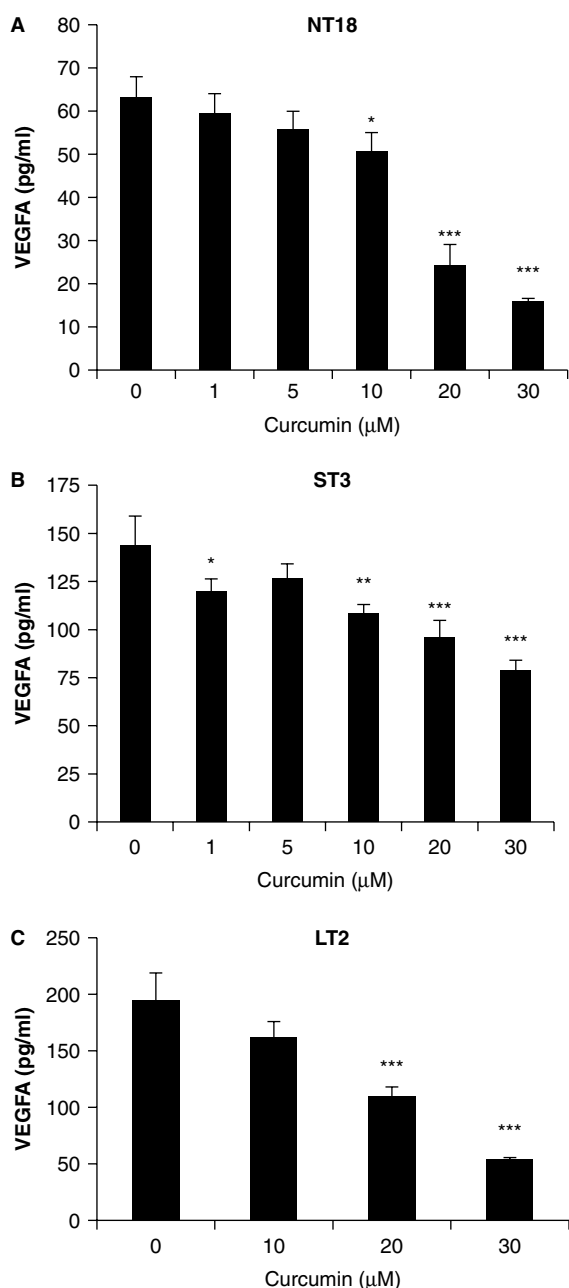
Figure 2 Effect of curcumin on the basal VEGFA release in three representative human pituitary adenoma cell cultures. Curcumin dose-dependently suppressed the VEGFA secretion in primary cell cultures of a non-functioning (A), a somatotroph (B) and a lactotroph (C) pituitary tumour. The cell cultures were treated with curcumin for 24 h in A and B and for 72 h in C. *$P<0.05$; **$P<0.01$; ***$P<0.001$ vs corresponding untreated cell cultures.

observed (Table 1). In some adenomas, in which the effect of different $CoCl_2$ doses on VEGFA secretion was studied, maximum effects were found with 125 μM $CoCl_2$ (data not shown) and therefore this dosage was used in all further adenoma investigated. As summarised in Table 1, VEGFA secretion was stimulated two- to tenfold under hypoxia-mimicking conditions. In general, a stronger induction was observed in those tumours with low basal VEGFA secretion rates. Curcumin dose-dependently suppressed the $CoCl_2$-induced VEGFA secretion not only after 24 h (Table 1; Fig. 5A, B, C and D) but also after prolonged treatment periods (48 and 72 h; Fig. 5A), and in several cases significant suppression was already achieved at 5 μM curcumin (Fig. 5B and C). In some cases, the highest doses of curcumin (30 μM) even completely abolished $CoCl_2$-induced VEGFA production (Fig. 5C). In summary, curcumin strongly suppressed not only basal but also $CoCl_2$-induced VEGFA secretion in all human endocrine-active and –inactive pituitary adenoma cell cultures studied.

Discussion

Anti-angiogenic treatment concepts targeting the VEGFA/ VEGFA receptor system are one of the most promising approaches for the treatment of different types of solid tumours (Wahl *et al.* 2011). It is thought that the resulting disturbance of tumour neovascularisation stops further tumour expansion and may probably induce tumour shrinkage. Curcumin, a polyphenolic compound of the spice plant *C. longa*, was shown to have anti-angiogenic properties in several types of tumours (Kunnumakkara *et al.* 2008, Yadav & Aggarwal 2011). Here, we could demonstrate that curcumin strongly suppresses the release of pro-angiogenic VEGFA under basal and hypoxia-mimicking conditions in rodent and human pituitary tumour cells and moreover inhibits HIF1A protein production.

HIF1A protein expression has been immunohistochemically demonstrated to be present in pituitary adenomas but not in normal anterior pituitaries (Vidal *et al.* 2003, Kim *et al.* 2005). Thus, as in any kind of solid tumour, it seems that pituitary adenoma neovascularisation is controlled by this transcription factor representing the major regulator of genes whose products are involved in angiogenic processes (Harris 2002, Hickey & Simon 2006). In cultured pituitary tumour cell lines or in primary cell cultures of human pituitary adenomas, we found *HIF1A* mRNA but not HIF1A protein under basal conditions because the latter on one hand is continuously synthesised but on the other hand is rapidly degraded by ubiquitination under normoxic conditions as they are present in cell culture (Wei & Yu 2007). Under hypoxic (incubation at 1% O_2) or hypoxia-mimicking ($CoCl_2$-treatment) conditions the degradation of HIF1A protein is suppressed and thus strongly increases in the pituitary tumour cells, reaching maximum values after 2–3 h, as we have reported previously (Shan *et al.* 2012). Curcumin potently inhibited HIF1A protein production both in rodent endocrine pituitary tumour cell lines and in cells of different human pituitary adenoma types. This has also been observed for curcumin and its derivatives in a variety of cell lines
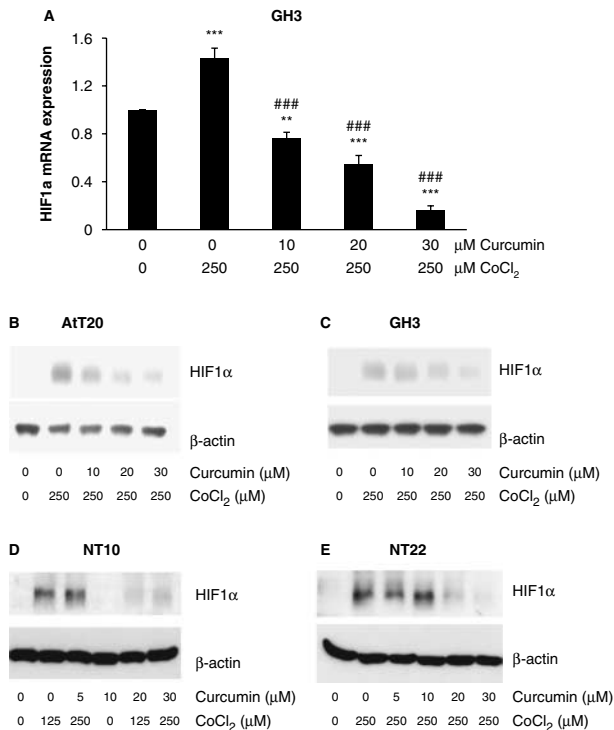
Figure 3 Effect of curcumin on HIF1A mRNA synthesis and protein production in pituitary tumour cells. (A) 24 h treatment with $CoCl_2$ (hypoxia-mimicking conditions) enhanced HIF1A mRNA synthesis. Increasing curcumin dosages not only reverted the $CoCl_2$-induced increase but also reduced basal HIF1A mRNA levels. Data are shown as relative mRNA expression in comparison to basal (=1). (B, C, D and E) Western blots showing the induction of HIF1A protein in response to $CoCl_2$ treatment and its suppression by curcumin in pituitary tumour cell lines (B and C) and representatively in cell cultures of two non-functioning human pituitary adenomas (D and E). For studies on HIF1A protein production, treatment periods with $CoCl_2$ were 3 h in each experiment. **$P < 0.01$; ***$P < 0.001$ vs basal HIF1A mRNA; ###$P < 0.001$ vs $CoCl_2$-induced HIF1A mRNA.

(Choi *et al.* 2006). In other cell types, curcumin concomitantly suppressed HIF1A and HIF1B (Ströfer *et al.* 2011). Whether the latter mode of curcumin-induced HIF1 suppression plays a role in pituitary tumour cells remains to be clarified in future studies.

The transcription factor HIF1 induces its angiogenic effects by stimulating different angiogenic factors, among which VEGFA is the most important. However, VEGFA also has other physiological functions such as maintaining the existing blood vessel system and regulating the vessel permeability by stimulating capillary fenestration (Ferrara 2004). An extremely dense intrapituitary vascular system (Viacava *et al.* 2003) and highly permeable capillary endothelial cells are essential for the rapid regulation and release of hormones within the anterior pituitary (Lafont *et al.* 2010). Therefore, VEGFA is already produced under normoxic conditions in the normal pituitary by FS cells, which are considered to be the major source of this factor in the adenohypophysis (Gospodarowicz *et al.* 1989, Gloddek *et al.* 1999). As curcumin was found to suppress VEGFA secretion by FS cells in the normal pituitary (Schaaf *et al.* 2010), it may attenuate the above-mentioned physiological functions of VEGFA, which needs to be clarified in future studies.
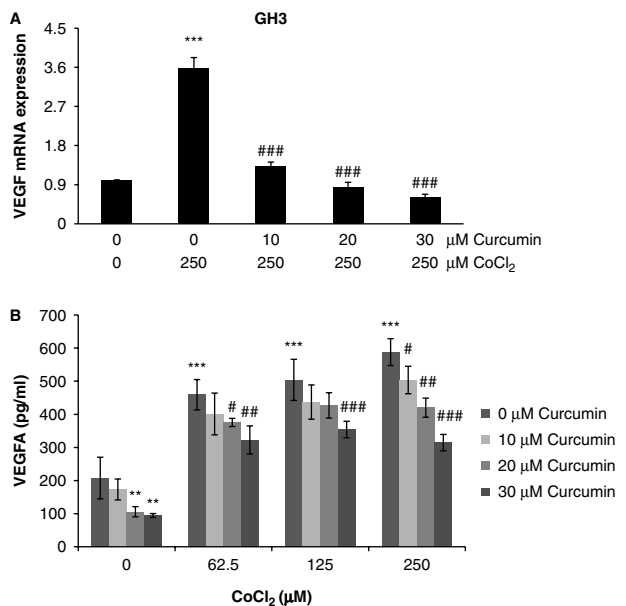

Figure 4 Influence of curcumin on $CoCl_2$-induced mRNA synthesis and secretion of VEGFA in GH3 cells. (A) Hypoxia-mimicking conditions (24 h treatment with $CoCl_2$) induced a strong increase in VEGFA mRNA synthesis in lactosomatotroph GH3 pituitary tumour cells that was completely abolished after application of increasing amounts of curcumin. (B) VEGFA secretion induced by different doses of $CoCl_2$ was also dose-dependently suppressed by curcumin, although the inhibition was less strong as for mRNA synthesis. **$P < 0.01$; ***$P < 0.001$ vs basal VEGFA mRNA synthesis or secretion respectively. #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ vs $CoCl_2$-induced VEGFA mRNA synthesis or secretion respectively.

serving as models of different types of tumours (Bae *et al.* 2006, Thomas *et al.* 2008, Jung *et al.* 2010, Ströfer *et al.* 2011). To the best of our knowledge, this is the first study demonstrating that curcumin also suppresses HIF1A protein production in primary culture of human tumour cells. In addition to protein suppression, curcumin could also completely inhibit hypoxia-induced *HIF1A* mRNA synthesis and even suppressed basal *HIF1A* mRNA production. Thus, curcumin not only interferes with the HIF1A protein generation and/or stabilisation system but also down-regulates HIF1A at the mRNA level, suggesting that different mechanisms of action mediate the overall inhibitory role of curcumin on HIF1A production. In a series of carcinoma cell lines, it has been shown that curcumin was not able to affect HIF1A production but suppressed HIF1 by down-regulating HIF1B (aryl hydrocarbon receptor nuclear translocator (ARNT)), the hypoxia-independent subunit of HIF1
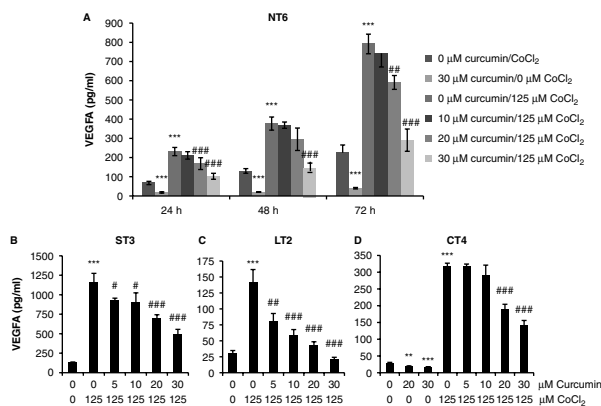
Figure 5 Influence of curcumin on $CoCl_2$-induced VEGFA secretion in human pituitary adenoma cell cultures. (A) The time-dependent increase in basal and $CoCl_2$-induced VEGFA secretion was dose-dependently suppressed by curcumin for each time period studied. (B, C and D) Dose-dependent inhibition of $CoCl_2$-stimulated VEGFA release by curcumin (24 h treatment period) in somatotroph (B), lactotroph (C) and corticotroph (D) adenoma cell cultures. $**P < 0.01$; $***P < 0.001$ vs corresponding basal VEGFA secretion. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs corresponding $CoCl_2$-induced VEGFA release.

In pituitary adenomas, FS cells are absent or rare and only very few cases of endocrine-inactive FS cell adenomas have been reported so far (Iwaki et al. 1986, Farnoud et al. 1994, Hori et al. 2009). Therefore, in most endocrine-active or -inactive pituitary adenomas, the tumour cells themselves are capable of producing VEGFA (Lloyd et al. 1999, Lohrer et al. 2001, Viacava et al. 2003). As shown here and in previous papers (Lohrer et al. 2001, Shan et al. 2012), cultured pituitary adenoma cells secrete VEGFA already under normoxic conditions in the absence of HIF1A. Thus, VEGFA production is in part an autonomous process or it is regulated by other factors (e.g. growth factors, cytokines and neuropeptides), many of which are produced within the tumour and may thus hypoxia-independently stimulate VEGFA by auto/paracrine mechanisms (Lohrer et al. 2001, Renner et al. 2004). This may explain why no correlation between the expression of VEGFA and HIF1A in pituitary adenomas was found in an immunohistochemical study (Kim et al. 2005).

Little is known in detail about the processes regulating pituitary tumour neovascularisation (Turner et al. 2000), but as in any solid tumour type, hypoxia may be the driving force (Harris 2002). We speculate that the basal VEGFA production is needed to maintain the already achieved intratumoural vasculature and to affect vessel permeability but may not be sufficient to induce angiogenesis. The latter may take place only transiently and locally in areas in which slowly growing pituitary adenoma cells have formed a not yet vascularised tumour cell population. If the oxygen supply of this cell population by diffusion declines, HIF1 production and VEGFA release will strongly increase to induce in concert

with other HIF1-induced angiogenic factors the neovascularisation of this adenoma cell population. This idea is supported by our findings that the basal VEGFA secretion of human pituitary adenoma cells was further strongly enhanced under hypoxia-mimicking conditions with the most prominent induction in adenoma cell cultures with low basal VEGFA secretion rates.

We could demonstrate that curcumin inhibited both basal and $CoCl_2$-induced VEGFA synthesis and secretion in pituitary tumour cells. In particular, the strong increase in VEGFA secretion under hypoxia-mimicking conditions in human adenoma cell cultures was in some cases completely reverted by curcumin, suggesting that this substance may have potent anti-angiogenic activities in pituitary adenomas. Most likely the suppressive effect of curcumin on HIF1A protein production was responsible for the reduction of VEGFA production and release. However, as the basal and HIF1A-independent release of VEGFA was also suppressed by curcumin, it is evident that other mechanisms involved in VEGFA production were also affected. It is known that curcumin has multiple targets and therefore interferes with multiple second messenger systems, intracellular signalling pathways and transcription factors (Kunnumakkara et al. 2008, Gupta et al. 2011), some of which are involved in the stimulatory action of different growth factors and neuropeptides on VEGFA secretion in pituitary adenomas (Renner et al. 2004). For instance, it has been shown that TGF-B-induced VEGFA production, a process that is also critically involved in pituitary tumour development (Renner et al. 2002), was significantly blocked by curcumin in human HT-1080 fibrosarcoma cells (Shih & Claffey 2001). Whether this mode of action of VEGFA suppression by curcumin and other mechanisms play a role in pituitary adenomas needs to be clarified still.

As we have previously shown, VEGFA affects not only vascular function and growth in pituitary adenomas but also stimulates proliferation of pituitary tumour cells (Onofri et al. 2006). These effects are mediated through differently localised VEGFA receptors, whereby the VEGFR1 is predominantly expressed on tumour cells and the VEGFR2 is exclusively located in endothelial cells (Onofri et al. 2006). Thus, through the inhibition of VEGFA, curcumin would not only impair the vascularisation of pituitary adenomas but would also suppress the growth stimulatory effects of VEGFA on pituitary tumours.

In summary, curcumin not only demonstrates the recently described anti-proliferative and pro-apoptotic effects in pituitary tumours but also displays anti-angiogenic properties by reducing the intratumoural production of HIF1A and VEGFA, which play important roles in angiogenesis-driven tumour neovascularisation. Therefore, if the problems of the poor bioavailability of curcumin can be solved, stable curcumin derivatives may be attractive candidates for the development of a multi-targeting therapy of pituitary adenomas.

Declaration of interest

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