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ORIGINAL ARTICLE

Post-transcriptional divergence in the regulation of CAT-2A, CAT-2B and iNOS expression by dexamethasone in vascular smooth muscle cells

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Abstract Upregulation of L-arginine transport by pro-inflammatory mediators is a widely reported phenomenon which accompanies the expression of the inducible nitric oxide synthase (iNOS) enzyme in various cells. Both processes require de novo protein synthesis which may be regulated differentially through diverging signalling pathways. This is particularly defined by observations that the glucocorticoid dexamethasone, acting potentially through NF-κB, selectively blocks the expression of iNOS whilst having little or no effect on transport; suggesting that this ubiquitous transcription factor may not be required for induced transporter activity. This notion is however controversial as is the suggestion that dexamethasone may regulate iNOS expression exclusively through NF-κB. Thus, to further understand the mechanisms that control these processes, we have examined the level at which dexamethasone acts, investigating whether this involves NF-κB and whether the latter selectively regulates iNOS induction. Our current data directly demonstrate that induced L-arginine transport is critically dependent on the activation of NF-κB, and further confirmed its role in the induction of iNOS in rat aortic smooth muscle cells. More importantly, dexamethasone enhanced both *iNOS* and *CAT* gene expression but repressed iNOS protein with no noticeable effects on transporter function or indeed NF-κB activation. These novel and unexpected findings reflect the complex nature of the regulation of iNOS by glucocorticoids and prove, contrary to previous assumptions, that dexamethasone can regulate *CAT* gene expression despite failing to alter transporter function. Moreover, the effects of dexamethasone occur through a non-NF-κB-mediated action even though NF-κB is required for both processes.

Keywords Inducible nitric oxide synthase · Dexamethasone · NF-κB · Cationic amino acid transporters · Smooth muscle cells · Septic shock

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Introduction

The induction of nitric oxide synthase (iNOS) in experimental models of endotoxin-induced shock occurs predominantly in the vascular smooth muscle layer of the vessel wall (Knowles et al. 1990) and is associated with significantly enhanced synthesis of nitric oxide (NO) which contributes to the pathogenesis of the disease state (Kilbourn and Griffith 1992). The sustained production of NO in this condition may be critically regulated by substrate supply (Schott et al. 1993a, b) through a family of monomeric cationic amino acid transporters (CATs); which in smooth muscle cells (SMCs) constitutes three members of the CAT family of proteins referred to as CAT-1, CAT-2A and CAT-2B (Baydoun et al. 1999). CAT-1, initially identified as the receptor for the murine ectropic retrovirus (Kim et al. 1991, Wang et al. 1991) and CAT-2B, originally cloned in endotoxin-activated macrophages (Closs et al. 1993a), show a low capacity and high affinity for L-arginine. CAT-2A, a spliced variant of CAT-2B (Closs et al. 1993b), exhibits the reverse profile (Closs et al. 2006).

There are strong indications that expression of the high affinity transporters within the vasculature plays an important regulatory role in determining the rate of NO synthesis since removal of exogenous L-arginine or direct inhibition of L-arginine transport through these carriers

attenuates NO synthesis in blood vessels obtained from endotoximic rats (Schott et al. 1993b) and in SMCs exposed to endotoxin and inflammatory cytokines (Hattori et al. 1999; Wileman et al. 2003). Moreover, knockout studies in mice have shown that suppression of L-arginine transport through abolition of CAT-2B results in a marked attenuation in NO production (Nicholson et al. 2001), demonstrating an important functional link between L-arginine transport and iNOS-mediated NO synthesis. This close coupling between the two processes may be of particular clinical relevance as induction of iNOS generally coincides with the induction of transporter activity. This phenomenon, demonstrated in in vitro cultured cell systems, including SMCs (Durante et al. 1995; Wileman et al. 1995; Hattori et al. 1999), in experimental models of endotoxin-induced shock in vivo (Hattori et al. 1999; Huang et al. 2004; Yang et al. 2005) and, more importantly, in patients with septic shock (Reade et al. 2002) provides a mechanism for sustained substrate supply during enhanced NO synthesis which may contribute to the pathogenesis of shock.

The induction of iNOS and L-arginine transport in SMCs requires gene expression and de novo protein synthesis but potentially diverging signalling pathways. The divergence in the intracellular mechanisms is reflected, in part, by the marked attenuation of induced NO synthesis but not of L-arginine transport following tyrosine kinase inhibition (Baydoun et al. 1999), and by the fact that the glucocorticoid dexamethasone selectively blocks iNOS expression but not transporter activity (Wileman et al. 1995). Since dexamethasone-induced suppression of iNOS in SMCs occurs through inhibition of the ubiquitous transcription factor NF-κB (Katsuyama et al. 1998; Matsumura et al. 2001), it is therefore likely that the differential regulation of iNOS expression and L-arginine transport in these cells occurs at a pre-transcriptional/transcriptional level, with NF-κB being critical for induction of the enzyme but not the transporters. There is, however, no published data in support of this notion in SMCs and the transcriptional events that lead to induction of L-arginine transport in these cells are currently unknown.

We have now carried out studies in rat cultured aortic smooth muscle cells to determine whether dexamethasone differentially regulates *iNOS* and *CAT* gene expression, and establishes whether such effects involve an inhibition of NF–κB activation. These studies have demonstrated an action of dexamethasone in SMCs that are not compatible with a selective suppression of NF-κB activation and indicate a complex regulation of both processes at a transcriptional and posttranscriptional level. In the first instance, dexamethasone showed little or no effect on NF-κB-DNA binding and, rather than suppress iNOS mRNA expression actually enhanced the latter whilst inhibiting iNOS protein expression. Moreover, despite its lack of effect on transporter activity, dexamethasone enhanced mRNA for both CAT-2A and CAT-2B but caused no change in CAT-1 mRNA expression.

Materials and methods

Cell culture

Smooth muscle cells were isolated from rat aortic explants and characterised as described previously (Wileman et al. 1995). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) plus 10% foetal bovine serum (FBS) and used between passages 3 and 6.

Measurement of L-[^{3}H] arginine transport and nitric oxide production

Confluent monolayers of cells in 96-well plates were incubated for 24 h with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) alone and in the presence of required drugs which were applied to the cultures either 30 min prior to or at 0, 1, 6 and 18 h after induction with LPS and IFN-γ. Nitric oxide production was monitored by measuring levels of accumulated nitrite in the culture medium using the Greiss assay, and L-arginine transport was measured at 37°C as described previously (Wileman et al. 1995).

Determination of changes in iNOS protein expression

Cells in 24-well plates were induced for 24 h with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) in the absence and presence of drugs which were added to the cells 30 min prior to activation. Lysates were prepared and samples resolved on a SDS–PAGE gel prior to detecting iNOS protein expression using a monoclonal anti-iNOS antibody (diluted 1:2,500) as described previously (Baydoun et al. 1999).

Assessment of cell viability

Controls and drug-treated cells were incubated with 0.5 mg ml^{-1} 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C. Formazan crystals produced were solubilised in 100 μl of isopropranol and absorbance readings taken at 550 nm on a Multiskan II plate reader. Cell viability under different experimental conditions was expressed relative to non-drug treated control cells.

Analysis of changes in transporter and iNOS mRNA expression

Total RNA was isolated from cells using RNA STAT-60 (Biogenesis, Poole, UK) according to the manufacturer's

protocol. Changes in the profile of expression of transporter and iNOS mRNA were determined using a one-step quantitative RT-PCR approach performed with the QuantiTect RT-PCR Kit (Qiagen). In these experiments, primers were designed for CAT-1, CAT-2A and CAT-2B. In all reactions, 0.5 μg total RNA was placed in a reaction mixture with 0.8 μM sense oligonucleotides (CAT-1: TCA CTG GCT GGA ACC TGA TTC; CAT-2A and -2B: GCC GCA GGC TCC CTC T) and antisense (CAT-1: CTC TCC GAT GGG TTT GCC TA; CAT-2A: TCT AAA CAG TAA GCC ATC CCG G; CAT-2B: CCA TCC TCC GCC ATA GCA TA). TaqMan hybridization probes (0.4 μM) dually labelled with 6-carboxyfluorescein (FAM) were used as the reporter fluorophore and carboxytetramethylrhodamine (TAMRA) as the quencher. The sequences used for each CAT were as follows: CAT-1: 6FAM-TCC TAC ATC ATC GGT ACA TCC AGC GTG GT XTp; CAT-2A: 6FAM-TTA CCC CGC ATT CTG TTT GCC ATG GTX Tp; CAT-2B: 6FAM-TGG ATC CAT TTT CCC AAT GCC TCG TXTp. The oligonucleotide sequence used for iNOS was: GAT GGG AAG CAT GAC TTC CG (sense), CAG GAT CCC CTC TGA TGG TG (antisense) and 6FAM-CCA GCT CAT CCG GTA CGC TGG CTA XTp (TaqMan probe). GAPDH was chosen as the housekeeping gene and detected using the following oligonucleotides: AAC TCC CTC AAG ATT GTC AGC AA (GAPDH sense), CTG TGG TCA TGA GCC CTT CC (GAPDH antisense) and 6FAM-CAT CCT GCA CCA CCA ACT GCT TAG CC XTp as the GAPDH TaqMan probe. To optimise the PCR reactions, dNTPs (400 μM) and $MgCl_2$ (5.3 mM) were included in the RT-PCR master mix. The RT phase of the reaction was allowed to run for 30 min at 50°C. The cDNA product was then heated to 95°C for 15 min and amplified through 50 cycles under the following conditions: denaturation at 94°C for 15 s, annealing and extension at 60°C for 60 s. Fluorescence was monitored at each 60°C annealing/extension step and recorded. The results obtained were analysed using the comparative Ct method (Livak and Schmittgen 2001) and presented graphically as relative expression.

Analysis of NF-κB activation by electrophoretic mobility shift assay (EMSA)

Confluent cells in T-75 flasks were either treated with DMEM alone or stimulated with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) for 5–180 min. In parallel studies, cells were pre-treated with dexamethasone (1 μM and 10 μM) or MG132 (0.3–3 μM) for 30 min prior to activation for 2 h. The latter was predetermined as the time point for peak NF-κB–DNA complex formation in our cell system. Nuclear extracts were subsequently prepared by resuspending cells in 500 μl of NP-40 lysis buffer [10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, 0.2 mM PMSF, 0.5 mM DTT, 10 μM sodium orthovanadate, 1.5 μg ml^{-1} of aprotinin, benzamidine, chymostatin, leupeptin and pepstatin, respectively] and centrifuging at 7,000 rpm for 5 min at 4°C. Following the removal of the supernatant, the pellet was gently resuspended in 500 μl buffer A [10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μM sodium orthovanadate and 1.5 μg ml^{-1} of aprotinin, benzamidine, chymostatin, leupeptin and pepstatin, respectively], vortexed and centrifuged at 7,000 rpm for another 5 min. The supernatant was again removed and the pellet resuspended in 50 μl of buffer B [10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF, 0.5 mM DTT, 10 μM sodium orthovanadate and 1.5 μg ml^{-1} of aprotinin, benzamidine, chymostatin, leupeptin and pepstatin, respectively]. The suspension was vortexed and agitated for 45 min at 4°C on a platform rotator and centrifuged at 13,000 rpm for 15 min. The supernatant containing the nuclear proteins was collected and mixed with 50 μl of buffer C [10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF, 0.5 mM DTT, 10 μM sodium orthovanadate and 1.5 μg ml^{-1} of aprotinin, benzamidine, chymostatin, leupeptin and pepstatin, respectively].

The activated status of NF-κB was determined as described by Torrie et al. (2001) using a synthetic oligomer (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) corresponding to the putative NF-κB binding site of the rat iNOS promoter region. The DNA probe (2 μl) was labelled with [γ-^{32}P] ATP (1 μl) in the presence of T4 polynucleotide kinase (1 μl), 5 μl of autoclaved H_2O and 1 μl of a 10× T4 polynucleotide kinase buffer (700 mM Tris–HCl pH 7.5), 100 mM $MgCl_2$, 50 mM DTT). Nuclear extracts (5–10 μg) were initially incubated on ice with 2 μl of a 5× binding buffer [50 mM Tris–HCl (pH 7.5), 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM and 0.25 mg ml^{-1} poly(dI–dC)]. Poly(dI–dC) for 10 min and then incubated further with 1 μl of radiolabelled probe for 40 min at 4°C before adding 1 μl of 10× loading buffer [250 mM Tris–HCl (pH 7.5), 0.2% bromophenol blue, 40% glycerol].

In some experiments, extracts were pre-incubated with a monoclonal anti-p65 antibody for 45 min prior to incubation with the labelled probe. This was carried out to show a super-shift in the detected band, confirming the presence of the p65 subunit and thus NF-κB as a major component of complex detected. In addition, a 100-fold excess of cold (unlabelled) oligonucleotide was added to some reactions to compete with labelled probe for DNA binding. The activation of NF-κB was subsequently analysed by resolving NF-κB–DNA complexes on a 5% polyacrylamide gel made with 5.2 ml H_2O, 5 ml of 50% autoclaved

glycerol, 14 ml of 4% bis-acrylamide, 750 µl APS, 50 µl TEMED and 5 ml of 10× gel running buffer and detecting the complexes by autoradiography.

Transfections of dominant negative IκB-α into RASMCs

Partially confluent (60–80%) monolayers of RASMCs were transfected with dominant negative IκB (DN-IκB) using an α5β1 integrin-binding polycationic peptide ($[K]_{16}$GACRRETAWACG; Peptide 6) as described previously (Cui et al. 2005). Briefly, 0.5 ml of transfection complex in Opti-MEM® I medium containing lipofectin (0.75 µl), plasmid (1 µg) and peptide 6 (4 µg) was added to cells in each well. The cells were then incubated for a further 2 h at 37°C and subsequently cultured in normal culture medium containing 10% FBS for 6, 12 or 24 h. Cell lysates were generated at the end of each time point and probed for IκB expression by western blot analysis.

Effects of dominant negative IκB-α on L-arginine transport, NO synthesis and iNOS expression

Control non-transfected and DN-IκB transfected RASMCs were activated for 24 h with LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}) at 6 h post transfection. Changes in NO production and L-arginine transport were determined as described above. The effects of DN-IκB on iNOS expression were determined by western blotting, using an anti-iNOS specific monoclonal antibody.

Materials

Cell culture reagents were obtained from Invitrogen (Paisley, UK). Dominant negative IKB-α plasmid was a gift from Robert Weil (Unite de Bilogie Moleculaire de l'Expression Genique, Institut Pasteur, Paris). Peptide 6 was synthesised by Sigma-Aldrich (Dorset, UK). The lipofectin used in all transfection studies was purchased from Life Technologies (Paisley, UK). The antibodies for IκB-α and p65 were purchased from Insight Biotechnology (Santa Cruz, Wembley, UK). Interferon-γ and MG132 were obtained from Calbiochem (Nottinghamshire, UK). Lipopolysaccharide from *E. coli*, stereotype 0111: B4 was purchased from Sigma-Aldrich (Dorset, UK). iNOS antibody and goat anti-mouse HRP were purchased from BD Transduction Laboratories (Oxford, UK). The synthetic NF-κB oligomer (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was from Promega (Southampton, UK). L-[2,3-^{3}H]arginine (36.1 Ci/mmol) and [γ-^{32}P]-ATP (3,000 Ci/mmol) were purchased from Amersham Life Science Ltd (Buckinghamshire, UK).

Statistics

All values are means ± SEM of measurements in at least three different cell cultures with three replicates per experiment. ANOVA was used to determine statistical significance between paired values with the overall confidence levels set at 95% ($p < 0.05$).

Results

Effects of dexamethasone on induced L-[^{3}H] arginine transport, NO synthesis and iNOS expression

Consistent with our previous studies (Wileman et al. 1995; Baydoun et al. 1999), exposure of RASMCs to LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}) resulted in the parallel induction of L-[^{3}H] arginine transport and NO production. Moreover, pre-treatment with dexamethasone (10 µM, 30 min) prior to activation failed to cause any significant change in either the basal or activated rates of L-arginine transport (Fig. 1a) but inhibited NO synthesis (Fig. 1b) and iNOS expression (Fig. 1c). Changes in CAT protein levels were not determined because of a lack of suitable commercially available antibodies. Time course experiments carried out to establish whether inhibition of induced NO synthesis was due to an action on the induction of iNOS, as opposed to a direct inhibition of the activity of the induced enzyme, revealed that dexamethasone was effective when added either prior to or for up to 1 h after treatment of cells with LPS and IFN-γ. In contrast, administration of dexamethasone at 6 h post activation caused significantly less inhibition while at 18 h there was no apparent inhibition when compared to control cells (data not shown).

Effects of dexamethasone on CATs and iNOS mRNA expression

To further explore the molecular mechanisms of action of dexamethasone, mRNA expression of CATs and iNOS was examined. Analysis of total RNA isolated from controls confirmed the presence of mRNA for CAT-1, CAT-2A and CAT-2B. More importantly, activation of cells with LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}) resulted in a marked reduction in CAT-1 but in a significant increase in both CAT-2A and CAT-2B mRNA expression. The increase peaked at 18 h and was maintained at the elevated levels over 24 h (data not shown). Parallel studies on iNOS expression revealed a similar time-dependent induction following exposure of cells to LPS and IFN-γ for periods of 1–24 h (data not shown).

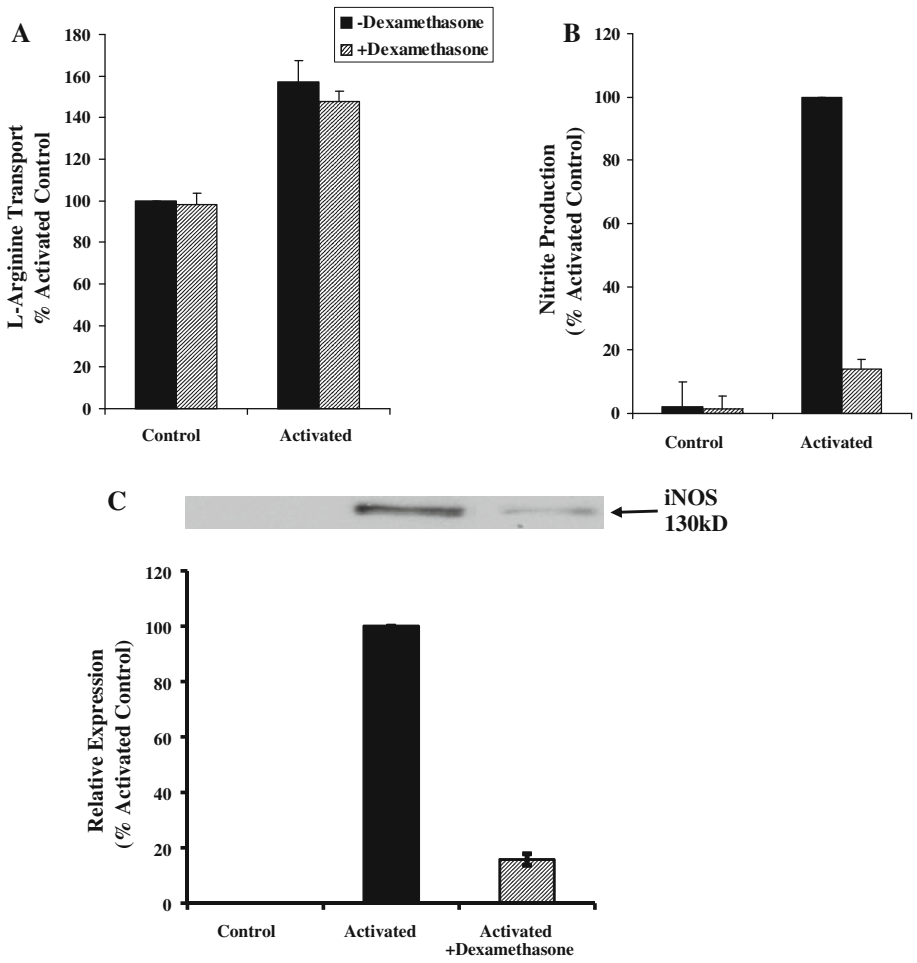

Fig. 1 Effects of dexamethasone on LPS and IFN-γ induced L-arginine transport, NO synthesis and iNOS expression. Confluent monolayers of RASMCs were activated with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) in the absence and presence of 10 μM dexamethasone. Nitrite production was measured after 24 h using the Griess assay (**b**) and transport of 100 μM L-[^{3}H] arginine (1 μCi ml^{-1}) by the underlying cell monolayers determined over 2 min (**a**). In parallel experiments, lysates were prepared and 20 μg of total cell protein analysed by western blotting as described in the methods using a monoclonal anti-iNOS antibody. Protein bands were quantified by scanning densitometry using a Bio Imaging System and the Gene Genius software program (**c**). Values for nitrite and transport are expressed as mean ± SEM of four experiments with five replicates in each. The densitometric data is expressed as a percentage of the activated samples

In both cases, pre-incubation of cells with dexamethasone (10 μM) for 30 min prior to activation caused a marked increase in CAT-2A (~3-fold), CAT-2B (~3-fold) and iNOS (~4-fold) mRNA expression above that seen in cells activated with LPS and IFN-γ alone (Fig. 2a, b). The decrease in CAT-1 mRNA was virtually unaltered by dexamethasone (Fig. 2a). These findings indicate clearly that dexamethasone enhances rather than inhibits *iNOS* gene expression. Moreover, this is not only restricted to the *NOS* gene but also genes that encode for CAT-2A and 2B, respectively. The latter observation is intriguing, since we have consistently demonstrated that induced transporter activity is insensitive to regulation by dexamethasone. These findings now question whether, in RASMCs, dexamethasone suppresses iNOS expression and induced NO synthesis through inhibition of NF-κB. We therefore investigated the regulation of NF-κB–DNA binding by dexamethasone and examined the role of NF-κB in the induction of both iNOS and CATs.

Effects of dexamethasone and MG132 on NF-κB activation

Initially, activation of NF-κB in RASMCs was confirmed by EMSAs, with exposure to LPS and IFN-γ inducing a time-dependent increase in NF-κB–DNA binding which reached a peak at 2 h (data not shown). This time point was therefore used in subsequent studies.

Incubation of cells with the proteasome inhibitor MG132 for 30 min prior to LPS and IFN-γ resulted in a concentration-dependent inhibition of NF-κB activation with 3 μM virtually abolishing NF-κB-DNA complex formation. The specificity of binding was confirmed in super-shift assays which showed the characteristic shift in the NF-κB-DNA complex when nuclear extracts were pre-incubated with a p65 selective antibody prior to initiating each reaction. Moreover, a 100-fold excess of unlabelled NF-κB-specific oligonucleotide completely abolished the bands produced using the labelled probe alone (Fig. 3a).

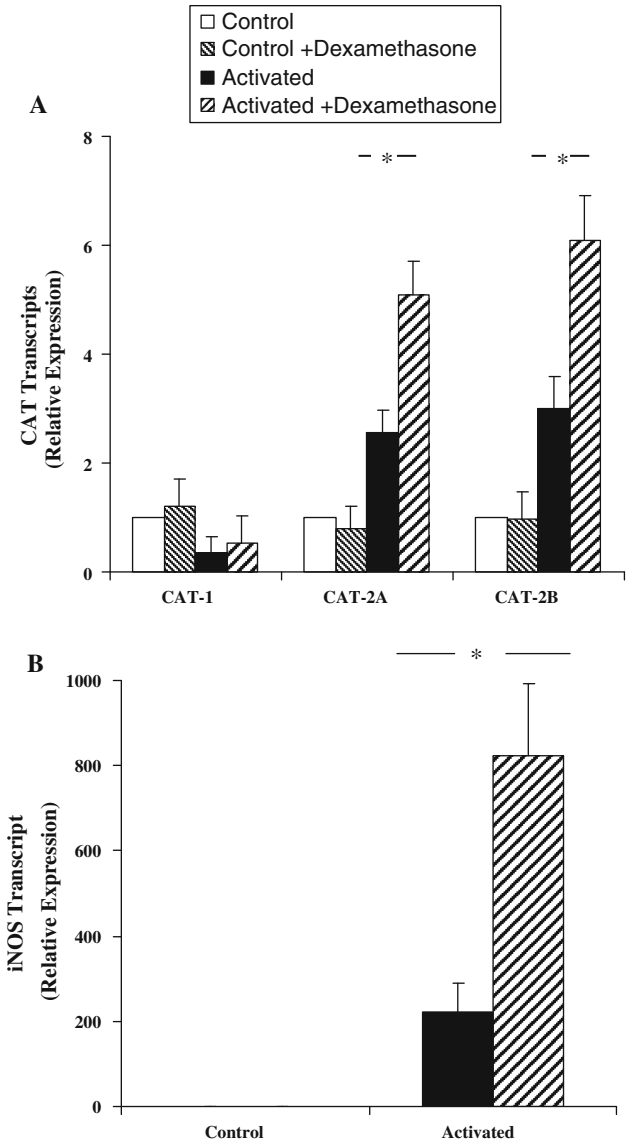

Fig. 2 Effects of dexamethasone on CAT and iNOS mRNA expression induced by LPS and IFN-γ. Confluent monolayers of RASMCs in 6-well plates were pre-treated with 10 μM dexamethasone for 30 min prior to activation with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) for 18 h. Controls were incubated in DMEM in the absence or presence of dexamethasone. Changes in levels of expression of CAT-1, CAT-2A, CAT-2B and iNOS mRNA were determined using a one-step quantitative RT-PCR protocol described in the methods. The data presented is expressed as the relative intensity of expression and is the mean ± SEM of three experiments. Statistical analysis was carried out using a One-way Anova followed by the Dunnett's Multiple Comparison Test. *$p < 0.05$ compared to activated cells alone

Unlike MG132, pre-treatment of cells for 30 min with dexamethasone (1 and 10 μM) failed to alter the increases in NF-κB-DNA binding observed at the peak 2 h time point (Fig. 3b) and this contrasts with the reports by Katsuyama et al. (1999) and by Matsumura et al. (2001). These observations question whether NF-κB is in fact

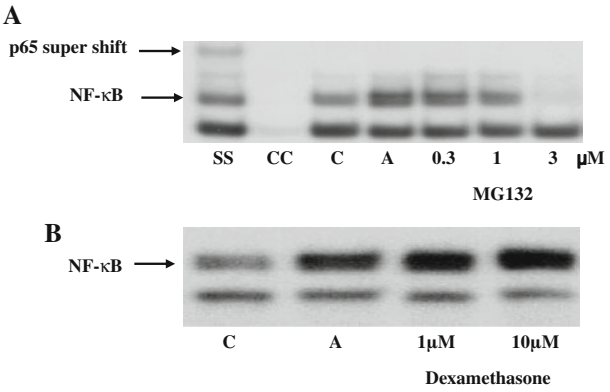

Fig. 3 Effects of MG132 and dexamethasone on LPS and IFN-γ induced NF-κB-DNA binding. Nuclear fractions were isolated from control cells and from cells activated with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) for 2 h in the absence and presence of MG132 or dexamethasone at the concentrations indicated. NF-κB-DNA binding was determined using 10 μg total nuclear protein in EMSAs as described in the methods. The autoradiograph is representative of three independent experiments. *C* and *A* represents nuclear fractions from control non-activated and activated cells respectively. SS shows super shift of P65 and *CC* represents cold competition

required for the induction of L-arginine transport and/or iNOS expression in our system.

Requirement of NF-κB for the induction of L-arginine transport and NO synthesis

Pre-incubation of RASMCs with MG132 (0.03–3 μM) caused a concentration-dependent inhibition of L-[^{3}H] arginine uptake but only in activated cells, with the control rates of transport remaining virtually unaltered even at the highest concentration (3 μM) used (Fig. 4a). In parallel with these findings, MG132 also caused a concentration-dependent inhibition of NO production with 3 μM reducing accumulated nitrite levels by ~75% from 0.46 ± 0.4 to 0.12 ± 0.01 nmoles μg protein^{-1} 24 h^{-1} (Fig. 4b). The compound had no adverse cytotoxic effects at the lower concentrations but significantly reduced cell viability at ≥10 μM (data not shown).

Transfection of cells with a plasmid encoding for dominant negative IκB-α resulted in the depletion of the endogenous IκB protein and an enhanced expression of DN-IκB-α which peaked at 6 h post transfection but declined over time, becoming barely detectable after 24 h (Fig. 5). Under these conditions, increased rates of L-arginine transport observed in activated cells were abolished while transport rates in controls remained virtually unaltered. The transfection mixture containing peptide 6 alone was without significant effect (Fig. 6a).

Consistent with the observations above, induction of NO production was also markedly attenuated in cells transfected with DN-IκB-α but virtually unaffected in cells

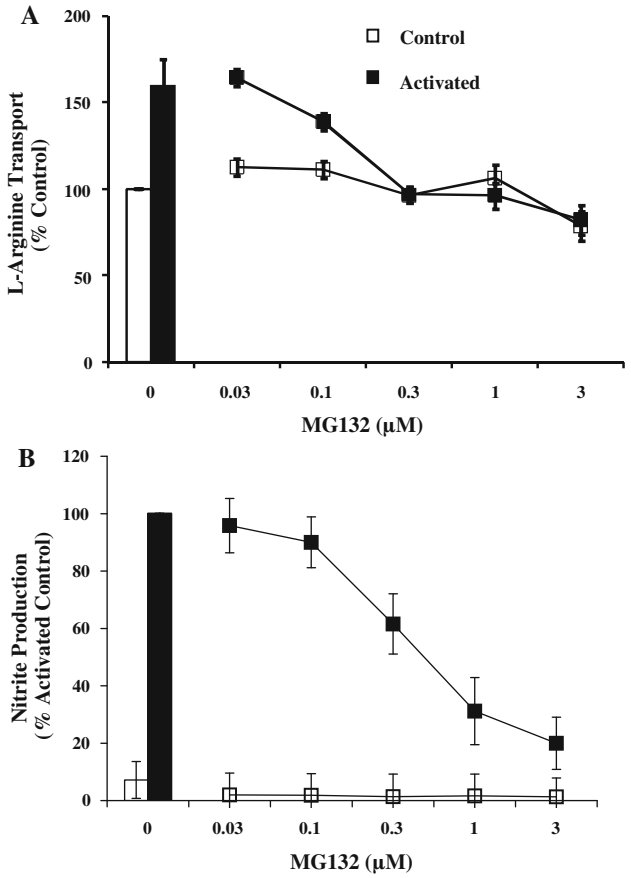

Fig. 4 Effects of MG132 on LPS and IFN-γ induced L-arginine transport and NO synthesis. Confluent monolayers of RASMCs in 96 well plates were activated with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) in the absence and presence of increasing concentrations of MG132. Nitrite production was measured after 24 h in the culture medium using the Greiss assay and transport of 100 μM L-[^{3}H] arginine (1 μCi ml^{-1}) by the underlying cell monolayer was determined over 2 min as described in the methods. Values are expressed as mean ± SEM of four experiments with five replicates in each

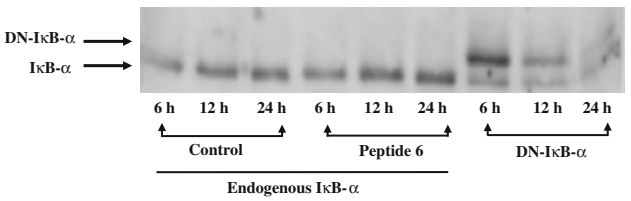

Fig. 5 Expression profile of transfected dominant negative IκB-α. Partially confluent monolayers of RASMCs were transfected with DN-IκB-α for the time points indicated. Lysates were subsequently generated and analysed by western blotting for IκB-α expression as described in the methods. The blot is representative of at least three independent experiments

incubated with peptide 6. Similarly, peptide 6 was without significant effect on basal nitrite production while DN-IκB caused a small but non-significant increase (~12%) (Fig. 6b). DN-IκB-α also significantly inhibited iNOS expression while peptide 6 alone had only marginal effects (Fig. 6c, d).

Discussion

The present study has shown the coordinated transcriptional regulation of *iNOS* and *CAT* gene expression in vascular smooth muscle cells, where both processes appear to be dependent on the activation of NF-κB, but with expression of the enzyme protein being selectively susceptible to inhibition by dexamethasone. Of particular interest is that the latter potentiated induced transcript levels for the enzyme and for the low affinity CAT-2A and the high affinity CAT-2B but not for the ubiquitious CAT-1 protein.

The detailed mechanisms associated with the increase of either CAT or iNOS transcripts in our studies are as yet unclear and merit further investigation. Conventionally, dexamethasone may exert its anti-inflammatory actions by binding to the inactive cytoplasmic glucocorticoid receptor leading to the activation of the latter and its subsequent translocation into the nucleus. Once in the nucleus, the activated receptor binds to the glucocorticoid-responsive element (GRE) in the promoter regions and initiates transcriptional activation or repression of the respective gene. This mechanism is however unlikely to account for the changes seen with transcripts for iNOS because there is no negative GRE in the promoter sequence of its gene (Adcock and Ito 2000). Whether dexamethasone, acting through GRE, is able to regulate CAT mRNA is also debatable since we currently do not know if the genes for these transporters have any GRE in their promoter regions.

In contrast to the vast literature on the regulation of the *iNOS* gene, very little is known about the regulation of CAT promoters and subsequent gene expression. These processes may be different for the various transporters, and our current observations show clearly that expression of CAT-1 mRNA is suppressed while that for CAT-2A and CAT-2B is enhanced following exposure of cells to LPS and IFN-γ. The potential mechanisms that mediate the latter changes involve the activation of NF-κB. In the first instance, EMSA analysis of nuclear extracts from LPS and IFN-γ activated cells showed a time-dependent increase in NF-κB–DNA complex formation, confirming the activation of the NF-κB pathway. More importantly, pre-treatment of cells with MG132 caused a concentration-dependent inhibition of induced transport of L-arginine and NO synthesis and these inhibitions occurred at concentrations that attenuated the activation of NF-κB. Further conclusive evidence was obtained in studies with DN-IκB-α transfected into RASMCs using peptide 6 which has an arginineglycine–aspartic acid (RGD) domain for binding to the

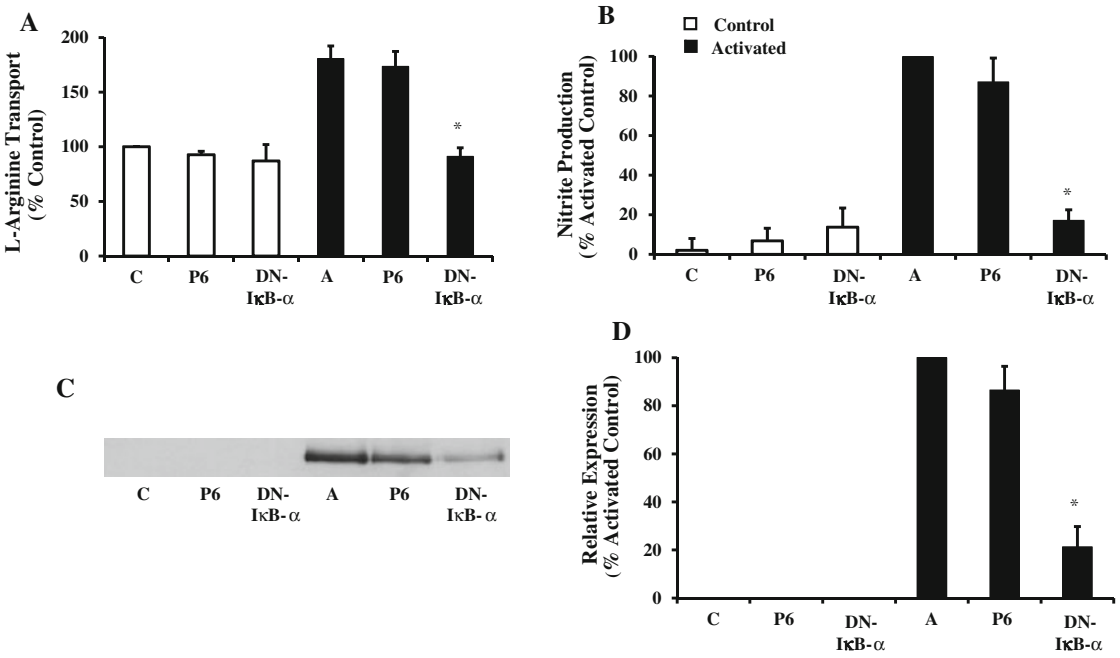

Fig. 6 Effects of dominant negative IκB-α on LPS and IFN-α induced L-arginine transport, NO production and iNOS expression. Partially confluent monolayers of RASMCs were transfected with DN-IκB-α for 6 h and subsequently activated with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) for 24 h. Accumulated nitrite levels in the culture medium (**b**) were determined by the Griess assay and transport of 100 μM L-[^{3}H] arginine (1 μCi ml^{-1}) by the underlying cell monolayer (**a**) was determined over 2 min. Expression of iNOS was determined in cell lysates by western blotting as described in the methods using a monoclonal anti-iNOS antibody (**c**). Protein bands were quantified by scanning densitometry (**d**) using a Bio Imaging System and the Gene Genius software program. Values for nitrite and transport are expressed as mean ± SEM of four experiments with five replicates in each. The densitometric data is expressed as a percentage of the activated samples. Statistical analysis was carried out using a One-way Anova followed by the Dunnett's Multiple Comparison Test. *$p < 0.05$ compared to activated cells

α5β1 integrins and a poly-L-lysine DNA binding tail (Hart et al. 1998). When used, peptide 6 produced a marked increase in the expression of the DN-IκB-α protein 6 h after transfection. Moreover, levels of endogenous IκB-α decreased quite markedly in the presence of DN-IκB-α, indicating that the latter may be the predominant suppressor of NF-κB in transfected cells. More importantly, expression of DN-IκB-α resulted in a significant reduction in induced transporter activity, NO production and iNOS expression but caused very little change in control non-induced cells. Taken together, the data suggest strongly that NF-κB, contrary to the original thinking, is critical for the induction of both iNOS and L-arginine transport in SMCs. This would be in agreement with findings in rat alveolar macrophages (Hammermann et al. 2000), human endothelial cells (Visigalli et al. 2004) and RAW264.7 macrophages (Lin et al. 2005) where transcriptional regulation of *CAT* genes through activation of NF-κB has been indicated but not conclusively proven.

The implication of NF-κB in the induction of iNOS is not surprising, as the promoter for this enzyme contains several binding sites for NF-κB and, moreover, its activation has been consistently reported to mediate enhanced expression of iNOS not only in SMCs (Katsuyama et al. 1998, 1999; Matsumura et al. 2001) but also in many other cell types including macrophages (Xie et al. 1994), hepatocytes (De Vera et al. 1997), glomerular mesangial cells (Eberhardt et al. 1994) and human lung epithelial A549/8 (Kleinert et al. 1996). In contrast, very little is currently known about the CAT promoters. There are indications however that the mouse *Cat-2* gene may contain two potential NF-κB sites (Finley et al. 1995). It is possible therefore that rat *CAT* genes also contain consensus binding sites for NF-κB which may be essential for gene transcription and this notion would be supported by our current findings.

Unlike MG132, dexamethasone did not cause any significant change in the activation of NF-κB indicating that it may not mediate its actions via the inhibition of the latter. This, however, contrasts with its reported action in the same cell type where it inhibits the activation of NF-κB through the suppression of IκB degradation at a concentration tenfold lower than the maximum ineffective concentration used in our study (Katsuyama et al. 1999). This study by Katsuyama et al. together with reports by Perrella et al. (1994) and Matsumura et al. (2001) also indicated that dexamethasone-induced inhibition of NF-κB in RASMCs prevented iNOS mRNA and protein expression,

contrasting with the complex regulation we have demonstrated at the transcriptional and posttranscriptional levels where iNOS mRNA is enhanced but protein expression inhibited.

The suppression of iNOS protein may be mediated at the induction level since dexamethasone was only effective when administered prior to or much earlier in the activation process. Thus, we can deduce that dexamethasone did not act directly on the enzyme but rather on the expression of the protein and this may be caused by an action at a post-transcriptional rather than at the transcriptional level. Although this was not addressed, likely targets for dexamethasone may include inhibition of the translation of mRNA and/or targeted degradation of the enzyme protein. These hypotheses now remain to be confirmed in RASMCs but would be consistent with a report in RAW 264.7 macrophages (Walker et al. 1997) and in rat mesangial cells (Kunz et al. 1996), where dexamethasone has been shown to attenuate iNOS expression by inhibiting mRNA translation and increasing degradation of the enzyme protein through activation of the cysteine protease, calpain I (Walker et al. 1997, 2001).

In summary, our current data have shown that induction of NO synthesis and L-arginine transport are both regulated in parallel by NF-κB and that gene expression of both iNOS and CATs (CAT-2A and CAT-2B) is enhanced by dexamethasone which subsequently represses iNOS expression but not the transporters. These novel and unexpected findings confirm the complex nature of the regulation of iNOS by glucocorticoids and prove, contrary to previous assumptions, that glucocorticoids can co-regulate *CAT* gene expression despite failing to alter transporter function. Moreover, dexamethasone may exert its effects at a site or sites further downstream of NF-κB which do not include (1) the destabilisation of CAT or iNOS mRNA expression, (2) inhibition of CAT mRNA translation and/or protein expression or, (3) the direct inhibition of iNOS or CAT protein activity. The point at which the NOS protein is regulated may be at the post-transcriptional level, potentially involving a decrease in translation and/or an increase in the degradation of the enzyme. These actions evidently do not apply to the CATs and as such the latter are insensitive to inhibition by dexamethasone.

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Conflict of interest The authors declare that they have no conflict of interest.

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