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Nuclear Factor-κB Mediates Simultaneous Induction of Inducible Nitric-Oxide Synthase and Up-Regulation of the Cationic Amino Acid Transporter CAT-2B in Rat Alveolar Macrophages

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ABSTRACT

The connection between the regulation of L-arginine transport and nitric oxide (NO) synthesis was studied in rat alveolar macrophages. Lipopolysaccharides (LPSs) and interferon-γ stimulated in the same concentration- and time-dependent manner NO synthesis (measured by nitrite accumulation) and L-[^{3}H]arginine uptake. This correlated with an increased mRNA expression for iNOS and the cationic amino acid transporter CAT-2B (analyzed by reverse transcription-polymerase chain reaction), with the same kinetics observed for the up-regulation of both mRNAs. Because nuclear factor-κB (NF-κB) is essential for induction of iNOS its role for the regulation of CAT-2B expression and L-arginine transport was investigated. The NF-κB inhibitors pyrrolidine dithiocarbamate and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone abrogated LPS- and interferon-γ-induced increase of nitrite accumulation and L-[^{3}H]arginine uptake as well as up-regulation of iNOS and CAT-2B mRNA. LPS-induced increase in iNOS and CAT-2B mRNA was also suppressed by specific NF-κB decoy oligodesoxynucleotides, confirming the essential role of NF-κB for iNOS and CAT-2B expression. Dexamethasone did not affect the initial (5 h) LPS-induced increase of iNOS and CAT-2B mRNA, but down-regulated both mRNAs after prolonged (20 h) exposure and this was accompanied by partial inhibition of LPS-stimulated nitrite accumulation and L-[^{3}H]arginine uptake. These findings demonstrate parallel regulation of the expression of iNOS and CAT-2B, and of NO synthesis and L-arginine uptake in rat alveolar macrophages. NF-κB is an essential transcription factor not only for the induction of iNOS, but also for the up-regulation of CAT-2B. The simultaneous up-regulation of CAT-2B with iNOS is considered as a mechanism to ensure a high substrate supply for iNOS.

Macrophages (MΦ) play an important role in host defense and immunity, and synthesis and release of nitric oxide (NO) are important elements of their machinery to fulfil these functions. Thus, NO is part of the effector mechanisms in the nonspecific defense (Moncada et al., 1991), but may also act as a signaling molecule to control inflammatory reactions (Brunn et al., 1997; Thomassen et al., 1997). Three isoenzymes of NO synthase (NOS) have been identified (Moncada et al., 1991; Förstermann and Kleinert, 1995) of which the inducible form (iNOS, also named NOS II) is responsible for NO production in MΦ, including alveolar MΦ (AMΦ) (Xie et al., 1992; Hey et al., 1995). Bacterial lipopolysaccharides (LPSs) and the cytokine interferon-γ (IFN-γ) are strong inducers of iNOS in MΦ and AMΦ (Sherman et al., 1993; Martin et al., 1994; Hey et al., 1995; Kim et al., 1995). Stimulation of NO synthesis in iNOS-expressing cells correlates usually with corresponding changes in iNOS mRNA levels, indicating that a major part of iNOS regulation occurs at the level of transcription (Förstermann and Kleinert, 1995). The promoter regions of the mouse (Lowenstein et al., 1993), human (Zhang et al., 1996), and rat (Eberhardt et al., 1996) iNOS genes have been characterized and contain several binding sites for transcription factors such as nuclear factor κB (NF-κB), activator protein-1, and various members of the CCAAT/enhancer-binding protein (C/EBP), activating

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ABBREVIATIONS: MΦ, macrophages; NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; AMΦ, alveolar macrophages; LPS, lipopolysaccharide; IFN-γ, interferon-γ; NF-κB, nuclear factor-κB; CAT, cationic amino acid transporter; ODN, oligodesoxynucleotide; PDTC, pyrrolidine dithiocarbamate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair.

transcription factor/cAMP response element binding protein (ATF/CREB), and signal transducer and activator of transcription (STAT) family of transcription factors. Among these transcription factor recognition sites only the activation of a downstream NF-κB site (Xie et al., 1994), the enhancer function of an upstream NF-κB site (Kim et al., 1997), and the activation of a novel lipopolysaccharide-response element, termed LRE_{AA} (Xie, 1997), have been shown to be involved in the expression of the iNOS gene in murine MΦ in response to LPS and/or IFN-γ. Finally, several lines of evidence indicate that NF-κB is an essential, although alone not sufficient transcription factor for the induction of iNOS in rat and mouse MΦ (Sherman et al., 1993; Xie et al., 1994; Ding et al., 1995; Kim et al., 1995; Xie, 1997).

Once expressed, iNOS is active and the amount of NO synthesized by MΦ depends critically on the availability of L-arginine (Baydoun et al., 1994; Hey et al. 1997; Hammermann et al., 1998). Induction of iNOS in MΦ and AMΦ is accompanied by an up-regulation of L-arginine uptake (Bogle et al., 1992; Hammermann et al., 1999). Different transport systems for the cellular uptake of L-arginine have been characterized, such as the cationic amino acid-specific, high-affinity transport system y^+ and several broad-scope amino acid transport systems (system y^+L, $b^{0,+}$, and $B^{0,+}$) (Closs, 1996; Devés and Boyd, 1998). System y^+ plays a particular role in MΦ and appears to mediate essentially the LPS-stimulated L-arginine transport (Bogle et al., 1992; Messeri Dreiβig et al., 2000). At the molecular level the transport properties of system y^+ could be ascribed to a family of cationic amino acid transporters (CATs) (Closs, 1996; Devés and Boyd, 1998). In rat and mouse AMΦ CAT-1 and a splicing variant of the CAT-2 gene (CAT-2B) were found to be expressed (Racké et al., 1998), and the LPS-induced up-regulation of L-arginine transport was accompanied by an increased expression of CAT-2B, but not of CAT-1 (Messeri Dreiβig et al., 2000).

The question arose whether the concomitant induction of iNOS and up-regulation of L-arginine transport and CAT-2B expression occurred via the same signal transduction pathway. Because NF-κB plays an essential role in the control of iNOS expression in MΦ, it was tested whether this signal transduction pathway is also involved in the up-regulation of L-arginine transport and the expression of CAT-2B in rat AMΦ, as a model of primary MΦ.

Experimental Procedures

Materials. Amphotericin B, L-arginine HCl, cycloheximide, deoxynucleotide mixture, dexamethasone, Dulbecco's modified Eagle's medium/nutrient mixture Ham's F-12, rat interferon-γ, lipopolysaccharides from *Escherichia coli* 0127:B8, mifepristone (RU-486), penicillin-streptomycin solution, pyrrolidine dithiocarbamate (PDTC), RedTaq DNA-polymerase, and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were all purchased from Sigma (Deisenhofen, Germany). L-[2,3-^{3}H]arginine HCl (1481 GBq/mmol) was purchased from DuPont (Dreieich, Germany), fetal calf serum from Vitromex (Vilshofen, Germany), DC Protein Assay from Bio-Rad (Munich, Germany), Trizol reagent for RNA isolation from Life Technologies (Karlsruhe, Germany), and avian myeloblastosis virus reverse transcriptase from Promega (Mannheim, Germany). All oligodesoxynucleotides for reverse transcription-polymerase chain reaction (RT-PCR) and decoy approach were obtained from MWG Biotech (Ebersberg, Germany).

Cell Preparation and Culture. Sprague-Dawley rats (own breeding) of either sex were killed by stunning followed by exsanguination. Lung and trachea were excised en bloc and lavaged three times by instilling 10 to 15 ml of cold PBS. Usually, for one preparation of AMΦ, lavage fluids from several lungs were pooled and centrifuged at 2500 rpm for 10 min. Thereafter, cells were resuspended in Dulbecco's modified Eagle's medium/nutrient mixture Ham's F-12 supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B, and plated in 12-well plates (10^6 AMΦ for uptake and NO accumulation studies), or 4×10^6 AMΦ were disseminated on 35-mm culture dishes (for RT-PCR and immunoblots). AMΦ were allowed to adhere for 2 h (37°C, 5% CO_2), before the medium was renewed to remove nonadherent cells. The adherent cells consisted of more than 95% AMΦ according to morphological criteria (May Grünwald-Giemsa staining). Thereafter, AMΦ were cultured for different time periods in the absence or presence of test substances.

L-[^{3}H]Arginine Uptake Studies. AMΦ were cultured for 5 to 20 h in the absence or presence of test substances. Subsequently, they were incubated at 37°C for 2 min in 0.5 ml L-[^{3}H]arginine (37 kBq, 100 nM) containing Krebs-HEPES solution [composition 118.5 mM NaCl, 5.57 mM KCl, 1.25 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.03 mM EDTA, 0.06 mM L-(+)-ascorbic acid, 20.0 mM HEPES (adjusted to pH 7.4 using NaOH) and 11.1 mM D-(+)-glucose]. Cells were lysed in 0.5 ml Tris/Triton (0.1%) solution followed by determination of the cellular radioactivity (Hey et al., 1997) and protein content using the DC Protein Assay (Bio-Rad). The 2-min incubation period was chosen because L-[^{3}H]arginine (100 nM) uptake was linear between 1 and 3 min (Racké et al., 1998). L-[^{3}H]arginine uptake was expressed either in absolute terms (picomoles per milligram of protein) or as percentage of the uptake observed in respective controls of the respective cell preparation.

Nitrite Assay. As a measure of NO synthesis during the culture period nitrite that accumulated in the culture media was determined. To estimate the iNOS activity after the 20-h culture period, the culture medium was removed and the AMΦ were incubated for 6 h in Krebs' solution containing 100 μM L-arginine. Nitrite was quantified by a spectrophotometric assay based on the Griess reaction as described previously (Hey et al., 1995). Briefly, 400 μl of Griess reagent [1% sulfanilic acid, 0.1% *N*-(1-naphthyl)ethylenediamine hydrochloride dissolved in 2.5% (w/v) H_3PO_4] was added to 400-μl incubation medium. After 20 min of incubation at room temperature absorbance was measured at 540 nm. The nitrite contents given under *Results* were calculated from a standard curve ($NaNO_2$) and expressed in absolute terms (nanomoles per 10^6 cells).

Immunoblotting of iNOS. Cellular proteins were extracted and separated by SDS-polyacrylamide gel (7.5%) electrophoresis (10 μg of protein per lane) and then transferred onto a polyvinylidene difluoride membrane. The immobilized proteins were visualized by subsequent incubation with a polyclonal rabbit antibody against mouse iNOS (Calbiochem, Bad Soden, Germany). A polyclonal horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as secondary antibody and staining was performed with the BM chemiluminescence blotting kit (Boehringer Mannheim, Mannheim, Germany). The "housekeeping" protein α-tubulin was detected with a monoclonal mouse anti-human α-tubulin antibody (Cedarlane, Hornby, Ontario, Canada), which shows cross-reactivity to rat α-tubulin and a secondary horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). Finally, the blots were exposed to Hyperfilm ECL (Amersham Buchler, Braunschweig, Germany).

Extraction of RNA and RT-PCR. Total RNA was isolated from AMΦ cultured for different time periods (up to 29 h) in the absence or presence of test substances and from freshly isolated AMΦ using Trizol reagent. The first strand cDNA was synthesized from 2 μg of total RNA using $Oligo(dT)_{18}$ primer and avian myeloblastosis virus reverse transcriptase under the conditions recommended by the supplier. The cDNA products were used for subsequent amplification by PCR. Oligonucleotide primers were constructed based on European

Molecular Biology Laboratory sequences for rat (r) β-actin (accession number V01217; J00691), riNOS (L12562), rCAT-1 (L10151), and rCAT-2B (U53927). Primer pairs were as follows: rβ-actin (612 bp), 5′-TTCTACAATGAGCTGCGTGTGGC-3′ and 5′-AGAGGTCTTTACGGATGTCAACG-3′; riNOS (525 bp), 5′-CATGAACTCCAAGAGTTTGACCAG-3′ and 5′-GCCCAGGTCGATGCACAACTGG-3′; rCAT-1 (769 bp), 5′-GCTGCCTCAACACCTATGATCTGG-3′ and 5′-ACGATGCCCACAGGAATGGC-3′; rCAT-2B (1050 bp), 5′-ATGGTGGCTGGGTTTGTGAAAG-3′ and 5′-CAACCCATCCTCCGCCATAGC-3′. PCR amplification was performed using RedTaq DNA polymerase and specific primers in a programmable thermal reactor (RoboCycler; Stratagene, Amsterdam, The Netherlands) with initial heating for 3 min at 94°C, followed by 25 (iNOS, CAT-2B), 35 (CAT-1), or 23–25 (β-actin) cycles of 45-s denaturation at 94°C, annealing at 56°C (30 s), extension at 72°C (1 min), and a final extension for 10 min at 72°C. PCR products were separated by a 1.2% agarose gel electrophoresis, documented by a video documentation system, and quantified by the RFLPscan software (MWG Biotech). In all PCRs a single amplification product of the expected size was obtained.

NF-κB Decoy Approach. Double-stranded ODNs were prepared from the complementary single-stranded partially phosphorothioate-bonded ODNs by melting at 95°C for 5 min followed by a cool-down phase of 3 h at room temperature. The double-stranded ODNs were preincubated with the cultured AMΦ after the 2-h adhesion period for 4 or 24 h at a concentration of 10 μM. Thereafter, 1 μg/ml LPS was added for 5 h followed by RNA isolation and RT-PCR. The single-stranded sequences of the NF-κB decoy ODN were as follows (underlined letters denote phosphorothioate-bonded bases): sense 5′-CTACTGGGACTCTCCCTTTG-3′ and that of the mutated ODN (bold letters mark mutations in the NF-κB consensus sequence) 5′-CTA**ACT**CTCT**GA**CTTTG-3′.

Statistical Analysis. All values are means ± S.E.M. of *n* experiments. Statistical significance of differences was evaluated by Student's *t* test or paired *t* test when appropriate. ANOVA followed by Dunnett's *t* test was performed when multiple test groups were compared with one control using the computer program GraphPad InStat (GraphPad Software, San Diego, CA).

TABLE 1

Effect of NF-κB inhibitors and dexamethasone on iNOS, CAT-2B and CAT-1 mRNA expression in rat AMΦ

AMΦ (4×10^6 cells/well) were cultured for 5 h in the absence (controls) or presence of 1 μg/ml LPS or 500 U/ml IFN-γ alone or in combination with 10 μM dexamethasone, 60 μM PDTC, or 100 μM TLCK. Thereafter, the cells were used for preparation of total RNA followed by RT-PCR with specific primers for rat iNOS, CAT-2B, CAT-1, and β-actin. The PCR products were separated on a 1.2% agarose gel and the optical densities of the PCR bands were determined by a video documentation system and the RFLPscan software. The bands were normalized by the following equation: sample value × 100/respective β-actin. Arbitrary units are presented as mean values ± S.E.M. of three to nine independent experiments. *$P < .05$ and **$P < .001$ compared with controls.

	iNOS	CAT-2B	CAT-1
		Absorbance	
		arbitrary units	
Controls	5.0 ± 1.7	25.6 ± 7.0	81.3 ± 4.1
LPS	69.7 ± 11.1**	76.2 ± 12.5*	74.5 ± 5.7
LPS + dexamethasone	76.0 ± 13.5**	90.2 ± 8.4*	81.4 ± 6.9
LPS + PDTC	8.7 ± 3.2	36.9 ± 12.2	84.0 ± 7.1
LPS + TLCK	24.7 ± 13.8	48.8 ± 15.9	68.8 ± 6.2
IFN-γ	58.0 ± 10.7**	45.8 ± 8.7*	71.4 ± 4.5
IFN-γ + dexamethasone	56.6 ± 14.2**	66.8 ± 11.3*	71.1 ± 6.0
IFN-γ + PDTC	1.5 ± 0.7	18.8 ± 4.0	79.1 ± 7.5
IFN-γ + TLCK	8.4 ± 5.8	18.9 ± 6.3	68.6 ± 6.7

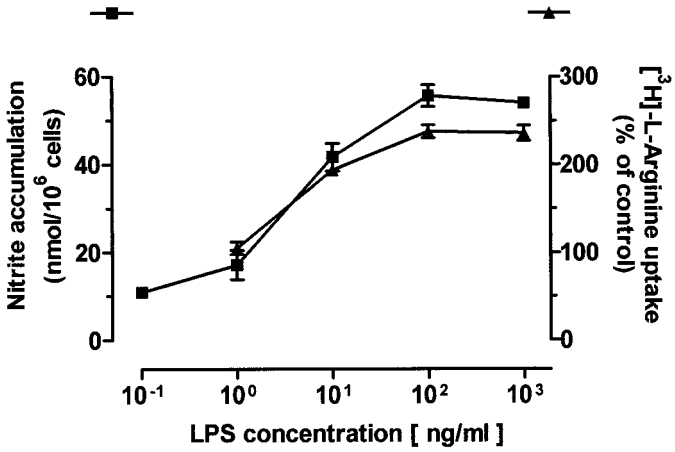

Fig. 1. Concentration-dependent effect of LPS on nitrite accumulation and L-[^{3}H]arginine uptake in rat AMΦ. Cells were cultured for 20 h under control conditions or with increasing concentrations of LPS as indicated by the abscissa. The medium was collected and analyzed for nitrite accumulation (left ordinate), whereas the cells were used to study L-[^{3}H]arginine uptake (37 kBq, 100 nM, 2 min) (right ordinate). Results are expressed in absolute terms for nitrite accumulation or as percentage of the controls of the respective cell preparation (for L-arginine uptake). Given are means ± S.E.M. of 6 to 21 experiments.

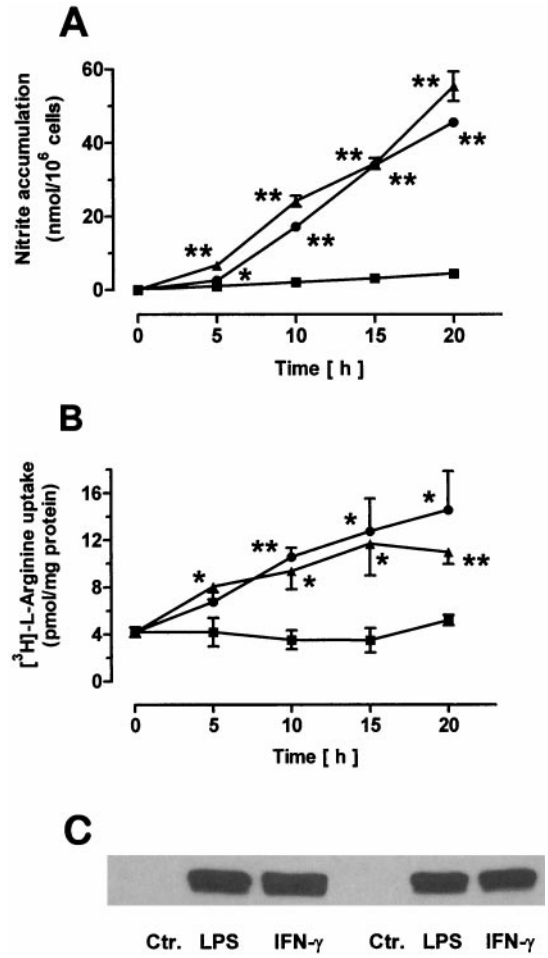

Fig. 2. Time-dependent effect of LPS and IFN-γ on nitrite accumulation and L-[^{3}H]arginine uptake in rat AMΦ. Cells were cultured under control conditions, with 1 μg/ml LPS or 500 U/ml IFN-γ for increasing time periods as indicated by the abscissa. The medium was collected and analyzed for nitrite accumulation (A), whereas the cells were used to study L-[^{3}H]arginine uptake (37 kBq, 100 nM, 2 min) (B). For A and B, control (■), 1 μg/ml LPS (▲), and 500 U/ml IFN-γ (●). Results are expressed as means ± S.E.M. of 6 to 14 experiments. *$P < .05$ and **$P < .01$ compared with the respective controls. C, examples of immunoblots for iNOS of two independent protein preparations from AMΦ cultured in the absence (Ctr.) or presence of 1 μg/ml LPS or 500 U/ml IFN-γ for 20 h are given.

Results

LPS- and IFN-γ-Evoked Up-Regulation of NO Production, L-Arginine Uptake, and mRNA Expression. Exposure of AMΦ to LPS or IFN-γ resulted in induction of iNOS mRNA (Figs. 3 and 7; Table 1) and protein (Fig. 2C), and an up-regulation of nitrite production (Figs. 1 and 2A). LPS and IFN-γ present for 20 h enhanced nitrite production in a concentration-dependent manner from about 1 to 2 to maximally about 50 to 60 nmol/10^6 cells/20 h at 1 μg/ml LPS (Figs. 1 and 2A) or 500 U/ml IFN-γ (data not shown; Fig. 2A). When cells were treated with maximally effective concentrations of LPS or IFN-γ for various time periods, nitrite accumulation was already significantly elevated after 5 h and increased continuously up to 20 h (Fig. 2A).

Treatment of LPS and IFN-γ resulted also in an increase of L-[^{3}H]arginine uptake from about 3 to 5 to maximally 10 to 15 pmol/mg of protein. Both the concentration dependence (shown only for LPS, Fig. 1) as well as the time course (Fig. 2B) of this effect were identical with the changes observed for nitrite accumulation.

As shown by RT-PCRs (Fig. 3), iNOS was not detected in freshly prepared AMΦ, but was slightly induced by the culture procedure alone. However, presence of LPS caused a rapid and persistent increase in iNOS mRNA, an effect seen already after 2 h and maximally expressed after 5 h. In freshly prepared AMΦ, mRNA for two cationic amino acid transporters, CAT-1 and CAT-2B, was detected. However, LPS caused a marked up-regulation of CAT-2B mRNA without significant effects on CAT-1 mRNA (Figs. 3 and 7; Table 1). The time course of up-regulation of CAT-2B mRNA paralleled that of iNOS mRNA (Fig. 3). When maximally effective concentrations of LPS and IFN-γ were present together for 20 h, their stimulatory effects on nitrite accumulation, L-arginine uptake, and mRNA expression of iNOS and CAT-2B were not additive (Fig. 4).

Effect of NF-κB Inhibitors on LPS- and IFN-γ-Induced NO Production, L-Arginine Uptake, and mRNA Expression. TLCK and PDTC, which had been shown to inhibit iNOS induction by neutralizing the NF-κB signal transduction pathway in many cells, caused similar effects in AMΦ. In a concentration-dependent manner both inhibitors decreased nitrite accumulation by about 60% when present alone and abrogated the LPS-induced up-regulation of NO production with the maximal effects seen at 100 μM TLCK and 60 μM PDTC for both basal and stimulated nitrite accumulation (Fig. 5A; data not shown). The IFN-γ-induced increase in nitrite accumulation was also prevented by 60 μM PDTC and largely inhibited by 100 μM TLCK (Fig. 6A).

Likewise, PDTC and TLCK reduced the basal L-[^{3}H]arginine uptake and abolished the LPS-induced up-regulation of L-[^{3}H]arginine uptake (Fig. 5B). A significant inhibitory effect of PDTC on basal L-[^{3}H]arginine uptake was, however, only observed when the data were expressed as a percentage of the respective controls of the individual cell preparations; PDTC (60 μM) caused a reduction by 30.2 ± 7.8% (n = 11, $P < .05$) and TLCK (100 μM) by 62.5 ± 4.8% (n = 9, $P < .01$). The stimulatory effect of IFN-γ was also prevented by PDTC and largely attenuated by TLCK (Fig. 6B).

In parallel, the influence of the NF-κB inhibitors on the mRNA expression of iNOS, CAT-2B, and CAT-1 was studied. Because the maximal induction of iNOS and CAT-2B mRNA was seen after 5-h exposure to LPS or IFN-γ, this time protocol was used to test the effects of the NF-κB inhibitors.

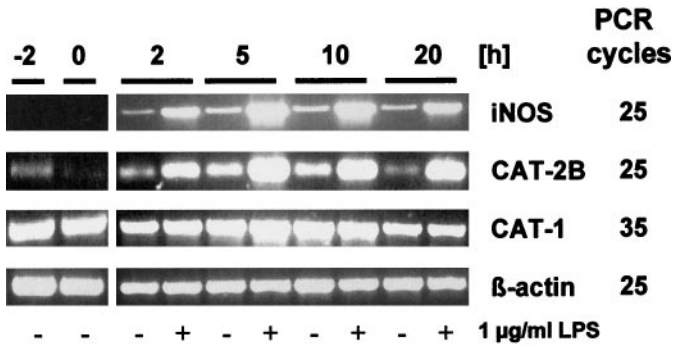

Fig. 3. Representative RT-PCRs performed with total RNA isolated from rat AMΦ. RNA was prepared from freshly isolated cells (−2 h), after the adhesion period (0 h), and after an additional culture period of 2, 5, 10, or 20 h in the absence or presence of 1 μg/ml LPS. After reverse transcription reaction the following PCRs were performed with specific primers for rat iNOS, CAT-2B, CAT-1, and β-actin. The PCR products were separated on a 1.2% agarose gel. The numbers on the right indicate the optimized PCR cycles. Shown is one of three similar experiments.

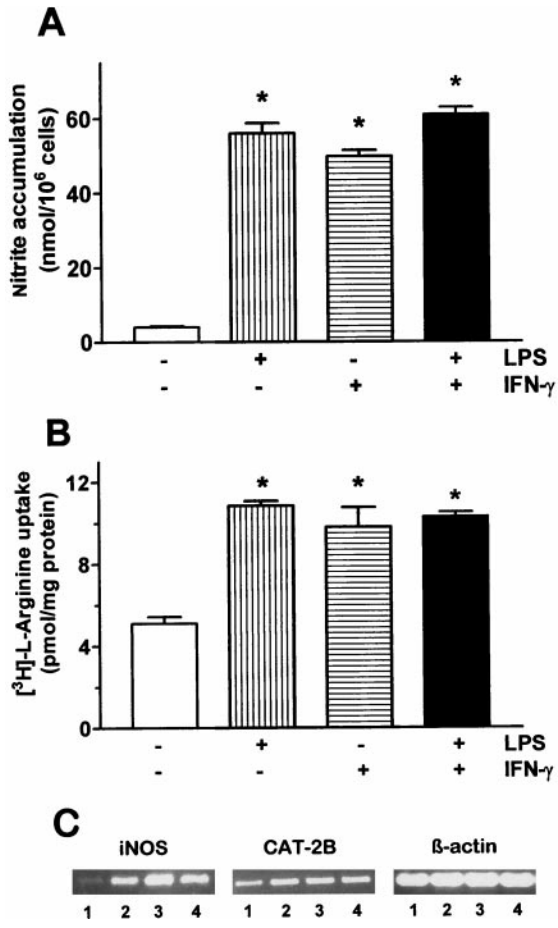

Fig. 4. Comparison of the effect of LPS and IFN-γ on nitrite accumulation, L-[^{3}H]arginine uptake, and mRNA expression for iNOS and CAT-2B in rat AMΦ. Cells were cultured under control conditions, with 1 μg/ml LPS and/or 500 U/ml IFN-γ for 20 h (A and B) or 5 h (C). The medium was collected and analyzed for nitrite accumulation (A), whereas the cells were used to study L-[^{3}H]arginine uptake (37 kBq, 100 nM, 2 min) (B). Results are expressed as means ± S.E.M. of three to nine experiments. *$P < .01$ compared with the respective controls. C, examples of RT-PCRs for CAT-2B, iNOS, and β-actin (one of three similar experiments). Lanes 1–4 represent controls, LPS-, IFN-γ-, and LPS plus IFN-γ-treated cells, respectively.

In these experiments LPS and IFN-γ caused a significant increase of the mRNA of iNOS and CAT-2B in the absence, but not in the presence of either PDTC or TLCK (Fig. 7; Table 1). The mRNA expression of the CAT-1 transporter was not significantly affected by any of these treatments (Fig. 7; Table 1). However, prolonged exposure (20 h) to the NF-κB inhibitors TLCK and PDTC caused a clear reduction in CAT-1 mRNA (data not shown).

Inhibition of NF-κB Signal Transduction Pathway by Decoy Approach. To confirm the involvement of NF-κB in the parallel induction of iNOS and CAT-2B, the decoy ODN technique was applied. Best results were obtained when the AMΦ were preincubated with 10 μM ODNs for 4 to 24 h and when 10 μM ODNs were additionally present during the incubation with the LPS stimulus. When present 4 h before LPS, the NF-κB decoy ODN reduced significantly the induction of iNOS and CAT-2B mRNA, whereas the mutated decoy ODN had no significant effect (Fig. 8; Table 2). After 24-h preincubation with the NF-κB decoy ODN (Table 2) the LPS-induced up-regulation of iNOS and CAT-2B mRNA expression was completely inhibited. However, 24-h preincubation with the mutated ODN caused also a significant reduction of the induced iNOS and CAT-2B mRNA, although the magnitude of this effect was substantially smaller than that of the NF-κB decoy ODN (Table 2). The prolonged exposure to the NF-κB decoy ODN resulted also in a significant reduction of the CAT-1 mRNA expression (Table 2), an observation that is in line with the above-mentioned effect caused by prolonged exposure to PDTC or TLCK. The long-term exposure to the mutated decoy ODN tended also to reduce the CAT-1 mRNA, but the NF-κB-specific decoy ODN caused a much larger and clearly significant effect (Table 2).

Effect of Cycloheximide on iNOS and CAT-2B mRNA Expression. Cycloheximide (10 μM), present 30 min prior and during the 5-h LPS stimulation prevented the induction of iNOS mRNA as well as the up-regulation of CAT-2B

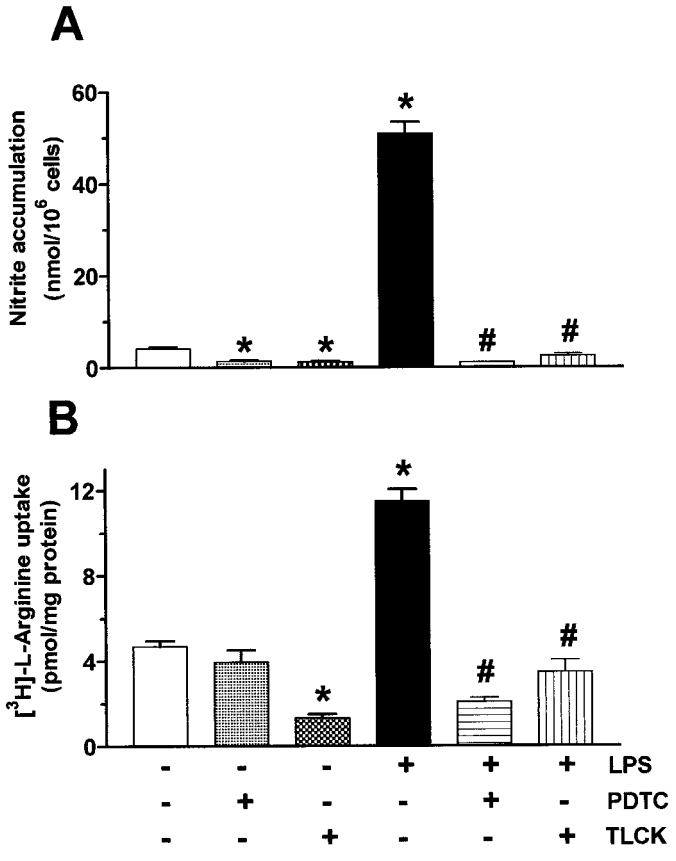

Fig. 5. Effect of NF-κB inhibitors and LPS on nitrite accumulation and L-[^{3}H]arginine uptake in rat AMΦ. Cells were cultured for 20 h in the absence (controls) or presence of 1 μg/ml LPS alone or in combination with 60 μM PDTC or 100 μM TLCK. Then the medium was collected and analyzed for nitrite accumulation (A), whereas the cells were used to study L-[^{3}H]arginine uptake (37 kBq, 100 nM, 2 min) (B). Results are expressed as means + S.E.M. of 6 to 26 experiments. $^*P < .0001$ compared with controls and $^{\#}P < .0001$ compared with LPS alone.

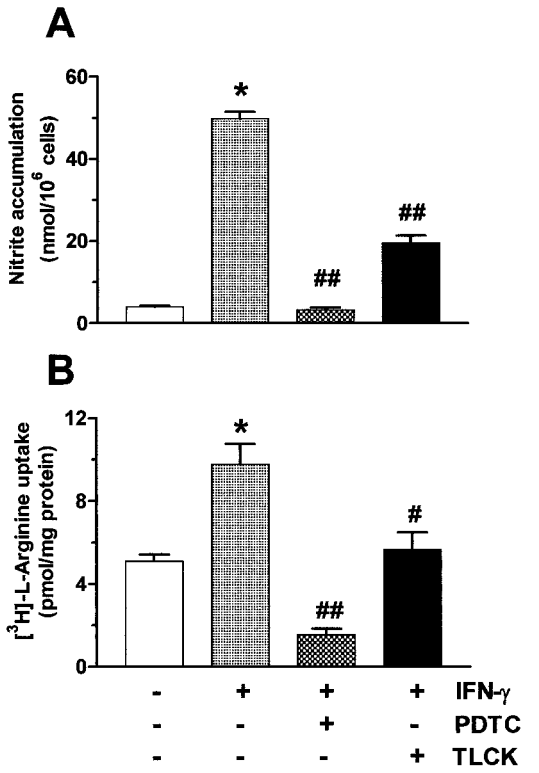

Fig. 6. Effect of IFN-γ and NF-κB inhibitors on nitrite accumulation and L-[^{3}H]arginine uptake in rat AMΦ. Cells were cultured for 20 h in the absence (controls) or presence of 500 U/ml IFN-γ alone or in combination with 60 μM PDTC or 100 μM TLCK. Then the medium was collected and analyzed for nitrite accumulation (A), whereas the cells were used to study L-[^{3}H]arginine uptake (37 kBq, 100 nM, 2 min) (B). Results are expressed as means + S.E.M. of 6 to 12 experiments. $^*P < .001$ compared with controls; $^{\#}P < .05$ and $^{\#\#}p < .0001$ compared with IFN-γ alone.

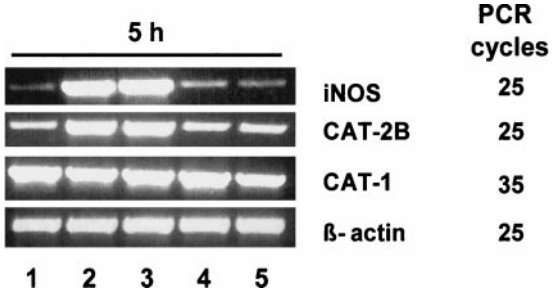

Fig. 7. Representative RT-PCRs performed with total RNA isolated from rat AMΦ. The RNA was isolated after a 5-h culture period under control conditions (lane 1), in presence of 1 μg/ml LPS alone (lane 2) or in combination with 10 μM dexamethasone (lane 3), 60 μM PDTC (lane 4), or 100 μM TLCK (lane 5). After RT-PCRs with specific primers for rat iNOS, CAT-2B, CAT-1, and β-actin the PCR products were separated on a 1.2% agarose gel. The numbers on the right indicate the optimized PCR cycles. For semiquantitative evaluation see Table 1.

mRNA (Fig. 9). The ratios of the absorbance of the PCR bands iNOS/respective β-actin expressed as percentage of the respective LPS value were 3 ± 4, 4 ± 2, and 3 ± 1% for controls, cycloheximide alone, and cycloheximide plus LPS, respectively. The respective values for CAT-2B were 42 ± 14, 39 ± 16, and 46 ± 12% (each $n = 3$, except for cycloheximide alone, $n = 2$). The expression of CAT-1 mRNA was not affected by cycloheximide, neither in the absence nor presence of LPS (data not shown).

Short- and Long-Term Effect of Dexamethasone on iNOS and CAT-2B Induction. Because glucocorticoids have also been shown to modulate iNOS expression, the effect of dexamethasone on nitrite accumulation, L-arginine uptake (Table 3), and the expression of mRNA for iNOS and the CATs (Figs. 7 and 10) in AMΦ was additionally studied. Presence of dexamethasone (0.1–10 μM) together with LPS during the 20-h culture period caused only a minor reduction of the induced nitrite accumulation (data not shown). Because nitrite determined in the medium of the 20-h culture period reflects NO synthesis over the whole culture period, additional experiments were performed to obtain information about the iNOS activity at the end of the 20-h culture period. For this, AMΦ were incubated for additional 6 h in Krebs-HEPES solution containing 100 μM L-arginine and the accumulation of nitrite was determined. As summarized in Table 3, AMΦ that had been cultured in the presence of LPS produced about 17 times more nitrite than control AMΦ. Additional presence of dexamethasone (0.1 and 1 μM) during the culture period resulted in a reduction of the LPS-induced NO synthesis by 45 and 55%, respectively. At the higher concentration of 10 μM, dexamethasone did not produce stronger inhibition (data not shown). Dexamethasone also reduced the basal nitrite accumulation, maximally by 28% (Table 3). The glucocorticoid receptor antagonist mifepristone (1 μM) prevented the effect of 0.1 μM dexamethasone and largely reduced that of 1 μM dexamethasone (Table 3).

Again, parallel changes were observed for L-[^{3}H]arginine uptake. Dexamethasone, in a concentration-dependent manner, diminished the basal level by 45%, and reduced the LPS-stimulated L-[^{3}H]arginine uptake by about 55% at 1 μM (Table 3). Like the effects on nitrite accumulation, mifepristone (1 μM) blocked the inhibitory effect of 0.1 μM dexamethasone and largely reduced that of 1 μM dexamethasone (Table 3).

In contrast to the NF-κB inhibitors, dexamethasone (up to 10 μM) did not affect the early LPS-induced increase in iNOS and CAT-2B mRNA (seen after 5 h, Fig. 7), but down-regulated the LPS-induced iNOS and CAT-2B mRNA expression after prolonged (20 h) exposure (Fig. 10) and tended to reduce the basal levels of these mRNAs. After 20-h culture, the ratios of the absorbance of the PCR bands iNOS × 100/respective β-actin were 5.5 ± 2.6 ($n = 11$) for controls, 0.5 ± 0.3 ($n = 6$) for dexamethasone alone ($P < .05$ versus controls), 69.9 ± 10.6 ($n = 8$) for LPS alone ($P < .001$ versus controls), and 1.6 ± 1.0 ($n = 3$) for LPS plus dexamethasone ($P < .001$ versus LPS alone). The respective values for CAT-2B were 14.5 ± 4.4 ($n = 11$) for controls, 5.0 ± 1.6 ($n = 6$) for dexamethasone alone ($P < .05$ versus controls), 96.8 ± 12.5 ($n = 8$) for LPS alone ($P < .001$ versus controls), and 32.9 ± 11.3 ($n = 3$) for LPS plus dexamethasone ($P < .01$ versus LPS alone). Again, the expression of CAT-1 remained unaffected

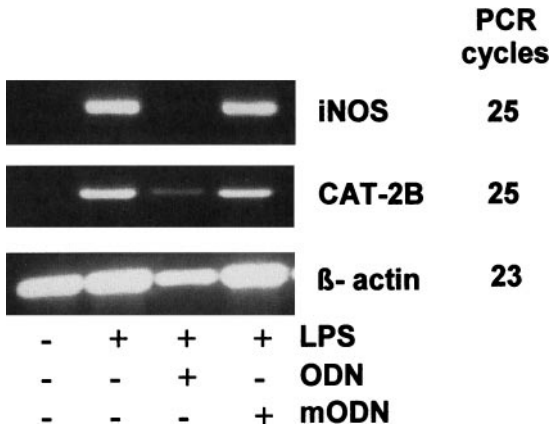

Fig. 8. Effect of NF-κB decoy ODN on the LPS-induced up-regulation of iNOS and CAT-2B mRNA in rat AMΦ. After a 4-h culture period in the absence or presence of 10 μM double-stranded NF-κB decoy ODN or the mutated ODN (mODN) the cells were cultured for additional 5 h in the absence or presence of 1 μg/ml LPS alone or in combination with NF-κB decoy ODN or mODN, as indicated. Then total RNA was isolated and RT-PCRs were performed with specific primers for iNOS, CAT-2B, and β-actin. The numbers on the right indicate the optimized PCR cycles. For semiquantitative evaluation see Table 2.

TABLE 2

Effect of NF-κB decoy ODN on iNOS, CAT-2B, and CAT-1 mRNA expression in rat AMΦ

AMΦ (4×10^6 cells/well) were cultured for 4 or 24 h in the absence (controls) or presence of 10 μM NF-κB decoy ODN or mutated ODN (mODN), followed by 5 h culture in the presence of 1 μg/ml LPS (each $n = 3$). Thereafter, the cells were used for the preparation of total RNA followed by RT-PCR with specific primers for rat iNOS, CAT-2B, CAT-1, and β-actin. For more details see Table 1. The ratio sample value ×100/respective β-actin of optical densities of the PCR bands are expressed as a percentage of the respective LPS value ± S.E.M. *$P < .05$ and **$P < .01$ compared with LPS alone.

	iNOS	CAT-2B	CAT-1
	% of respective value with LPS		
4 h + 5 h			
Controls	5.2 ± 2.1**	24.7 ± 5.6**	86.5 ± 10.1
LPS	100	100	100
ODN + LPS	39.8 ± 17.1*	37.6 ± 12.7*	75.1 ± 21.9
mODN + LPS	69.9 ± 18.1	78.9 ± 15.2	98.3 ± 11.1
24 h + 5 h			
Controls	9.8 ± 4.9**	32.1 ± 15.2**	110.3 ± 19.4
LPS	100	100	100
ODN + LPS	3.8 ± 1.4**	11.6 ± 2.1**	24.3 ± 5.0**
mODN + LPS	67.2 ± 11.2*	52.0 ± 15.4*	74.3 ± 6.2

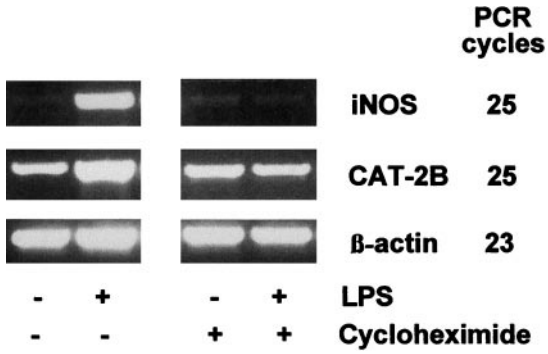

Fig. 9. Effect of cycloheximide on the LPS-induced up-regulation of iNOS and CAT-2B mRNA in rat AMΦ. AMΦ were first cultured for 0.5 h in the absence or presence of 10 μM cycloheximide and then for 5 h in the absence or presence of 1 μg/ml LPS and/or 10 μM cycloheximide. Thereafter, total RNA was isolated and RT-PCRs were performed with specific primers for iNOS, CAT-2B, and β-actin. The numbers on the right indicate the optimized PCR cycles. For semiquantitative evaluation see text.

TABLE 3

Effect of glucocorticoids on nitrite accumulation and L-[^{3}H]arginine uptake in rat AMΦ cultured in the absence or presence of LPS

AMΦ (10^6 cells/well) were cultured for 20 h in the absence (controls) or presence of 1 μg/ml LPS, mifepristone, and dexamethasone at the indicated concentrations. Thereafter, the medium was removed and the cells were used for L-[^{3}H]arginine uptake studies (for details see *Experimental Procedures*). In parallel, AMΦ were additionally incubated for 6 h in Krebs-HEPES solution containing 100 μM L-arginine, and nitrite accumulated in the solution was measured. Data are expressed in absolute values for nitrite accumulation or as a percentage of the controls of the respective cell preparation for L-[^{3}H]arginine uptake. Given are means ± S.E.M. of 6 to 27 experiments. *$P < .05$ and **$P < .001$ compared with respective value in the absence of dexamethasone; #$P < .05$ and ##$P < .001$ compared with the respective value in the absence of mifepristone.

	Dexamethasone		
	0	0.1	1
		μM	
Nitrite accumulation (nmol/10^6 cells)			
6-h incubation after 20-h culture			
Controls	1.1 ± 0.1	1.0 ± 0.1	0.8 ± 0.1*
LPS	17.4 ± 0.8	9.6 ± 0.6**	7.9 ± 0.5**
LPS + 1 μM mifepristone	n.d.	15.7 ± 0.3##	12.8 ± 0.7##
L-[^{3}H]arginine uptake (% of controls)			
2-min uptake after 20-h culture			
Controls	100.0 ± 1.1	72.2 ± 13.6**	55.9 ± 3.8**
LPS	279.4 ± 8.8	178.4 ± 11.8**	150.7 ± 8.5**
LPS + 1 μM mifepristone	n.d.	298.2 ± 29.1##	220.0 ± 34.0#

n.d., not determined.

for all tested time periods and concentrations of dexamethasone (Fig. 7; Table 1; data not shown).

Discussion

In confirmation of previous observations on different types of MΦ (Bogle et al., 1992; Hammermann et al., 1999) the present results demonstrated that induction of iNOS in rat AMΦ by LPS or IFN-γ is accompanied by an activation of L-arginine transport. Moreover, both the LPS- and IFN-γ-mediated induction of NO synthesis and the activation of L-arginine transport occurred in the same time- and concentration-dependent manner (Figs. 1 and 2), suggesting already that induction of iNOS and up-regulation of L-arginine transport might be mediated by the same signal transduction pathways.

As outlined in the Introduction, there is evidence that most of the cellular uptake of L-arginine in rat AMΦ, particularly in LPS-stimulated cells, is mediated via specific, high-affinity CATs (Messeri Dreiβig et al., 2000). Of the five known members of the CAT family CAT-1 and CAT-2B have been shown to be expressed in rat AMΦ, but only the expression of CAT-2B was enhanced after exposure to LPS or IFN-γ (Table 1), suggesting that an increased availability of the CAT-2B transporter may be responsible for the enhanced L-arginine uptake. Similar observations have been made in different murine MΦ cell lines (Closs et al., 1993; Kakuda et al., 1999). Moreover, the up-regulation of CAT-2B mRNA occurred simultaneously with the iNOS mRNA induction (Fig. 3), giving further support to the idea of a parallel regulation of iNOS expression and L-arginine transport.

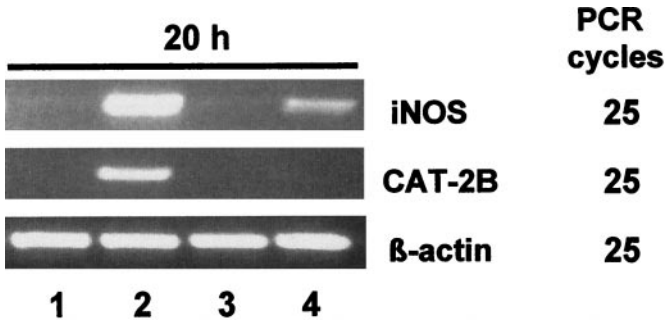

Fig. 10. Representative RT-PCRs performed with total RNA isolated from rat AMΦ. The cells were cultured for 20 h under control conditions (lane 1), with 1 μg/ml LPS (lane 2), 10 μM dexamethasone (lane 3), or LPS in combination with dexamethasone (lane 4). Thereafter, RNA was isolated and RT-PCRs with specific primers for rat iNOS, CAT-2B, CAT-1, and β-actin were performed. The PCR products were separated on a 1.2% agarose gel. The numbers on the right indicate the optimized PCR cycles. For semiquantitative evaluation see text.

Because NF-κB is an essential transcription factor for the induction of iNOS in MΦ (see Introduction), this transcription factor appeared to be a likely candidate also for the regulation of CAT-2B expression. Although at present the promoter region of the rat CAT-2 gene is not yet known, we could identify two NF-κB sites in the partially clarified mouse CAT-2 gene promoter (Finley et al., 1995) using the program MatInspector version 2.2 (Quandt et al., 1995). The present experiments applying three different strategies to inhibit NF-κB-induced gene transcription indicate that indeed NF-κB is not only involved in the regulation of NO synthesis by inducing the expression of iNOS but also in the regulation of L-arginine transport by controlling the expression of CAT-2B. PDTC and TLCK, which inhibit NF-κB activation by acting at different sites in the NF-κB signaling pathway (Sherman et al., 1993; Kim et al., 1995), concomitantly abrogated LPS- and IFN-γ-induced NO synthesis and iNOS expression on one side and the up-regulation of L-arginine transport and CAT-2B expression on the other side (Figs. 5–7; Table 1). Because the specificity of PDTC as antioxidant and TLCK as serine protease inhibitor with regard to NF-κB inhibition might be questionable, we finally used the transcription factor decoy approach to demonstrate unequivocally the involvement of NF-κB in the regulation of CAT-2B expression. ODN decoy approaches have been applied successfully to inhibit transcriptional activation of different genes in different cell types, including NF-κB-activated genes in MΦ (von Knethen et al., 1999). Treatment of rat AMΦ with the NF-κB decoy ODN, of which the susceptibility to nuclease degradation had been reduced by introducing phosphorothioate nucleotides, caused a parallel attenuation of the LPS- and IFN-γ-induced increase of iNOS and CAT-2B mRNA (Fig. 8; Table 2). The specificity of the effect of the NF-κB decoy ODN was demonstrated by parallel experiments with an ODN in which the NF-κB consensus sequences had been mutated. When the AMΦ were preincubated with the ODNs 4 h before the LPS stimulus, the NF-κB

decoy ODN caused a clear reduction of both the induced mRNA of iNOS and CAT-2B, whereas the mutated ODN had no significant effect. When the ODNs were present already 24 h before the LPS stimulus, the induction of iNOS mRNA and the up-regulation of CAT-2B mRNA were prevented by the NF-κB decoy ODN. However, under these conditions, the mutated ODN caused also a significant, although substantially smaller, reduction of the induced iNOS and CAT-2B mRNA (Table 2). Whether this is the result of weak interactions with NF-κB or caused by unspecific effects, possibly due to the phosphorothioate nucleotides, remains unclear. Nevertheless, the marked effects of the NF-κB-specific decoy ODN together with those of TLCK and PDTC strongly support the conclusion that NF-κB is an essential transcription factor not only for induction of iNOS but also for the up-regulation of CAT-2B. Interestingly, prolonged inhibition of the NF-κB pathway, by either TLCK, PDTC, or the NF-κB decoy ODN resulted also in a clear reduction of the CAT-1 mRNA, suggesting that the expression of CAT-1 may in part be driven by a background activity of NF-κB.

It had been shown that NF-κB is an essential, but alone not sufficient transcription factor for the induction of iNOS in murine MΦ cell lines (Xie et al., 1994, Ding et al., 1995) and the same is true for the activation of the iNOS and CAT-2 genes in rat AMΦ. The observation that cycloheximide prevented the up-regulation of iNOS and CAT-2B mRNA indicates that in addition to NF-κB that is present in the cytosol as inactive complex with different isoforms of inhibitor of NF-κB (Baeuerle, 1998), newly synthesized proteins contribute to the activation of the respective promoters. The nature of such proteins remains unknown at present.

Finally, a closely parallel modulation of iNOS expression and NO synthesis on one side and CAT-2B expression and L-arginine transport on the other side was also observed for glucocorticoids. Dexamethasone did not affect the initial up-regulation of iNOS and CAT-2B mRNA (Fig. 7; Table 1), but caused a down-regulation of both mRNAs after prolonged exposure (Fig. 10). After 20-h exposure to dexamethasone the LPS effect on iNOS mRNA was abolished and that on CAT-2B mRNA largely attenuated. The magnitude of the effect of dexamethasone on mRNA levels appeared to be larger than that on the functional responses, particularly for the inhibition of iNOS mRNA and the reduction of stimulated NO synthesis. This apparent discrepancy can be explained by a delayed decline of iNOS protein after the down-regulation of the de novo synthesis. The inhibitory effects of dexamethasone on NO synthesis and L-arginine transport were antagonized by the glucocorticoid receptor antagonist mifepristone (Cadepond et al., 1997), indicating a nuclear site of action via the glucocorticoid-receptor complex. However, the delay in onset of the effects of dexamethasone argues against a direct suppressive action at the promoter region of the iNOS and CAT-2 gene. An indirect modulation via the induction of inhibitory *trans*-acting factors such as lipocortin-1 (Flower and Rothwell, 1994), inhibitor of κB (Scheinman et al., 1995), or E4BP4 (Wallace et al., 1997) appears to be more likely. However, this has not been illuminated in the present study. It should be mentioned that experiments with antibodies against lipocortin-1 indicated that this glucocorticoid-induced protein may at least in part mediate the inhibitory effects of glucocorticoids on iNOS induction in murine MΦ (Wu et al., 1995). The indirect way of action of glucocorticoids may also explain some of the cell-specific differences in their effects. Thus, in murine J774 MΦ (Baydoun et al., 1993) and astrocytes (Schmidlin and Wiesinger, 1995) glucocorticoids inhibited LPS-mediated iNOS induction, but not LPS-induced up-regulation of L-arginine transport. However, cell type-specific differences in the regulation of the expression of CATs appear also possible, because it was very recently shown that in J774 MΦ the LPS-induced up-regulation of CAT-2B and iNOS mRNA occurred with different kinetics (Closs et al., 2000), in contrast to the present observations on rat AMΦ.

In conclusion, the present findings demonstrated a parallel regulation of the expression of iNOS and CAT-2B, and of NO synthesis and L-arginine uptake in rat AMΦ. NF-κB was shown to be an essential, but alone not sufficient transcription factor for both the induction of iNOS and the up-regulation of CAT-2B. The simultaneous up-regulation of CAT-2B with iNOS is considered as a mechanism to ensure a high substrate supply for iNOS. A functionally close link between CAT-2B-mediated L-arginine transport and iNOS-mediated NO synthesis has recently been demonstrated in MΦ of mice in which the CAT-2 gene was ablated (MacLeod et al., 1999), because in MΦ from CAT-2 knockout mice the induction of iNOS did not result in an increased NO synthesis.

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