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Effects of phosphodiesterase inhibitors on L-arginine pathways in rat alveolar macrophages

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Abstract

Effects of phosphodiesterase inhibitors on L-arginine-dependent pathways in rat alveolar macrophages, inducible nitric oxide (NO) synthase (iNOS) and arginase, were studied. Culture of rat alveolar macrophages in the presence of lipopolysaccharides (20 h) caused an increase of arginase activity (by 135%) and nitrite concentration (fourfold). The nonselective phosphodiesterase inhibitor IBMX (2-isobutyl-1-methylxanthine) enhanced arginase activity by 35% and nitrite accumulation by 130%. IBMX caused a clear increase in iNOS protein levels and a relatively smaller increase in iNOS mRNA. The effect of IBMX on nitrite accumulation was largely attenuated by the protein kinase A inhibitor K 5720. The phosphodiesterase 4 inhibitor rolipram enhanced nitrite accumulation more effectively than the phosphodiesterase 3 inhibitor siguadozan (about 50% and 20% of IBMX effect, respectively), whereas the phosphodiesterase 3/4 inhibitor benzafendrine was as effective as IBMX. In conclusion, in rat alveolar macrophages, phosphodiesterase 4 and, to a smaller extent, phosphodiesterase 3 play a role in the control of iNOS-mediated NO synthesis.

Keywords: Alveolar macrophage; Nitric oxide synthesis; Phosphodiesterase; Lipopolysaccharide; Arginase; iNOS

1. Introduction

In cells of the monocyte/macrophage lineage, L-arginine pathways play a particular role in host defense mechanisms and the control of inflammatory reactions (e.g. Moncada et al., 1991; Nozaki et al., 1993; Xie and Nathan, 1994; Barnes and Liew, 1995; Brunn et al., 1997; Wu and Morris, 1998). Arginase, which catalyses the formation of L-ornithine and urea, appears to be expressed constitutively in macrophages, although its expression and activity may be modulated by various stimuli such as endotoxins and different cytokines (e.g. Hey et al., 1995; Modolell et al., 1995; Wang et al., 1995; Corraliza et al., 1995, 1997; Sonoki et al., 1997; Louis et al., 1998; Morris et al., 1998; Klasen et al., 2001). Although the endothelial nitric oxide synthase (eNOS) has been detected in alveolar macrophages (Miles et al., 1998), the inducible isoenzyme (iNOS), induced by bacterial toxins and/or pro-inflammatory cytokines (e.g. Jorens et al., 1991; Xie and Nathan, 1994; Hey et al., 1995; Wang et al., 1995; Hammermann et al., 1998, 2000b), is responsible for the large amounts of nitric oxide (NO) released by these cells.

Between iNOS and arginase, multiple interactions can occur, suggesting counter-regulatory functions of these two pathways. Thus, L-arginine metabolism by arginase can limit L-arginine utilization by iNOS (Hey et al., 1997; Chang et al., 1998; Tenu et al., 1999), but on the other hand, iNOS by liberating N^G-OH-L-arginine can exert inhibitory effects on arginase (Hecker et al., 1995; Buga et al., 1996). Arginase may also limit L-arginine availability for the constitutive isoforms of NOS, and functional experiments indicate that arginase by limiting NO synthesis may contribute to the development of airway hyperreactivity (Meurs et al., 2000, 2002).

Phosphodiesterases, of which at least 11 heterologous families of isoenzymes are known, are involved in regulating the dynamic equilibrium of cyclic nucleotides (cAMP and/or cGMP). Phosphodiesterase inhibitors by causing a rise in intracellular cAMP levels have a number of potentially anti-inflammatory effects and play an increasing role in the treatment of chronic inflammatory obstructive airway diseases (Torphy, 1998; Schmidt et al., 1999; Schudt et al., 1999; Torphy and Page, 2000). Since in macrophages including alveolar macrophages the cAMP–protein kinase A

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(PKA) pathway is involved in the regulation of both L-arginine-dependent pathways (Corraliza et al., 1997; Chen et al., 1999; Morris et al., 1998; Hammermann et al., 2000a), the present study aimed to characterize the effect of phosphodiesterase inhibitors on L-arginine pathways in rat alveolar macrophages.

A preliminary report of part of the present experiments has been given (Koschorreck et al., 2002).

2. Materials and methods

2.1. Preparation and culture of alveolar macrophages

Sprague–Dawley rats (own breeding) of either sex were killed by stunning followed by exsanguination. Lung and trachea were excised en bloc, washed with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (D-PBS) and lavaged three times by 10–15 ml of cold (4 °C) D-PBS (see Hey et al., 1995). Usually, for one preparation of alveolar macrophages, lavage fluids of four to six lungs were pooled and centrifuged at 400 × *g* for 5 min. The cells were washed with D-PBS and thereafter resuspended in Dulbecco's modified Eagle's (DME)/F-12 medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B, and plated in sterile 12-well dishes (10^6 alveolar macrophages per well, for nitrite accumulation studies and arginase assay) or 35-mm dishes (5×10^6 alveolar macrophages per well, for reverse transcription-polymerase chain reaction (RT-PCR) and immunoblots) (NUNC, Wiesbaden, Germany). The alveolar macrophages were allowed to adhere for 2 h (37 °C, 5% CO_2). Thereafter, the medium was renewed to remove non-adherent cells. The adherent cells consisted of more than 95% alveolar macrophages according to morphological criteria (May Grünwald-Giemsa staining). Furthermore, no epithelial cells could be detected by staining with a cytokeratin(pan)-antibody as described by Freitag et al. (1996). Cells were cultured for 5–20 h in the absence or presence of 1 μg/ml lipopolysaccharides and/or other test substances. Cell viability was assessed by trypan blue exclusion and was always about 95%.

2.2. Nitrite accumulation studies

As a measure of NO synthesis during the culture period, nitrite, which accumulated in the culture media, was determined. Nitrite was quantified by a spectrophotometric assay based on the Griess reaction as described previously (Hey et al., 1995). Briefly, 400-μl 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 concentrations given under Results were calculated from a standard curve ($NaNO_2$) and expressed in absolute terms (μM concentration in the culture medium). In each cell preparation, experiments were run in triplicate and the mean values considered to reflect one independent experiment.

2.3. Arginase assay

For the determination of arginase activity, cell lysates were prepared after the culture period by incubating the cells in 750-μl 0.1% Triton X100 containing additionally the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupetin (0.5 μg/ml), pepstatin A (0.7/μg ml) and EDTA (2 mM). Thereafter, 500-μl portions of the cell lysates were mixed with 500-μl 25 mM Tris HCl (pH 7.4) containing 5 mM $MnCl_2$ and incubated for 10 min at 56 °C. Then 50 μl of a 0.5 M L-arginine solution (pH 9.7) was added to 50 μl of the heat-activated lysates and incubated for 1 h at 37 °C. The amounts of urea were then determined by a spectrophotometric assay based on a reaction with α-isonitrosopropiophenon (Corraliza et al., 1995). The arginase activity was calculated from a standard curve (urea) and was expressed in absolute terms (U/mg protein; one unit is the enzyme activity which catalyzes the formation of 1 μmol urea/min) or as percentage of the mean value of the respective controls of the individual cell preparation.

2.4. Extraction of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared and the first strand cDNA synthesized as described previously (Hammermann et al., 1998; Racké et al., 1998). Oligonucleotide primers were constructed based on EMBL sequences: rat β-actin, 5′-TTCTACAATGAGCTGCGTGTGGC-3′ and 5′-AGAGGTCTTTACGGATGTCAACG-3′; rat iNOS, 5′-CATGAACTCCAAGAGTTTGACCAG-3′ and 5′-GCCCAGGTCGATGCACAACTGG-3′; rat arginase I, 5′-AAAGCCCATAGAGATTATCGGAGCG-3′ and 5′-AGACAAGGTCAACGGCACTGCC-3′; rat arginase II, 5′-TTAGTAGAGCTGTGTCAGGTGGC-3′ and 5′-ACTTGAAGCAATCACATCCACTGC-3′. PCR amplification was performed using RedTaq DNA polymerase and specific primers in a programmable thermal reactor (RoboCycler®, Stratagene, Amsterdam, Europe) with initial heating for 3 min at 94 °C, followed by 25 (iNOS, arginase I and β-actin) or 35 (arginase II) 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 and documented by a video documentation system and the optical density of the bands was quantified by the RFLPscan 2.01 software (MWG Biotech, Ebersberg, Germany). PCR products were sequenced (MWG, single strand analysis) and showed 99% homology to the respective published cDNA sequence (Klasen et al., 2001).

2.5. Immunoblotting of iNOS

Cellular proteins were extracted and separated by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) (7.5%; 10 μg protein per lane) and then transferred onto a polyvinylidene difluoride (PVDF) membrane as described previously (Hammermann et al., 2000b; Klasen et al., 2001). 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 immunoglobulin G (IgG) (BioRad, Munich, Germany) was used as secondary antibody, and staining was performed with the BM chemiluminescence blotting kit (Boehringer Mannheim, Germany). The 'housekeeping' protein α-tubulin was detected with a mouse monoclonal anti-human α-tubulin antibody (Cedar Lane, Hornby, Canada) and a secondary horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad). Finally, the blots were exposed to Hyperfilm ECL (Amersham Buchler, Braunschweig, Germany). Optical density of the bands was quantified by using a video documentation system and the RFLPscan software.

2.6. 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, if applicable. When several treated groups were compared with one control, the significance of differences was evaluated by the modified t-test according to Dunnett using the computer program GraphPad InStat (GraphPad Software, San Diego, CA, USA). $P<0.05$ was accepted as being significant.

2.7. Drugs and special chemicals

Amphotericin B, L-arginine HCl, desoxynucleotide mixture, Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DME/F-12 medium), IBMX (2-isobutyl-1-methylxanthine, Biomol, Hamburg, Germany); 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) (Alexis Deutschland, Grünberg, Germany); α-isonitrosopropiophenon, leupeptin; lipopolysaccharides from *Escherichia coli* 0127:B8, penicillin–streptomycin solution, pepstatin A, phenylmethylsulfonyl fluoride, RedTaq DNA polymerase (all Sigma, Deisenhofen, Germany); benzafendrine (AH 21-132, Novartis, Basel, Switzerland); DC Protein Assay (BioRad); fetal calf serum (Biochrom, Berlin, Germany); rolipram (Schering. Berlin, Germany); siguazodan (SKF 94836, SmithKline Beecham Pharmaceuticals, Worthing, UK); Trizol® reagent for RNA isolation (Life Technologies, Karlsruhe, Germany); and AMV reverse transcriptase (Promega, Mannheim, Germany). All oligodesoxynucleotides for RT-PCR were obtained from MWG Biotech.

Stock solutions were prepared in distilled water, dimethyl sulfoxide (IBMX, siguazodan) or ethanol (rolipram). Dimethyl sulfoxide or ethanol alone at the maximum concentrations (1% and 0.1%, respectively) had no effect on nitrite accumulation and arginase activity in alveolar macrophages (not shown).

3. Results

3.1. Effects of lipopolysaccharides and IBMX on arginase activity and nitrite accumulation

The basal arginase activity determined in rat alveolar macrophages after a 20-h culture period under control conditions was 0.39 ± 0.05 U/mg protein ($n=9$). Generally, arginase activity showed much smaller variations within one cell preparation than between different cell preparations. Therefore, in the following, the data are mainly expressed as percent of the controls within an individual cell preparation. As in previous studies (Klasen et al., 2001), lipopolysac-

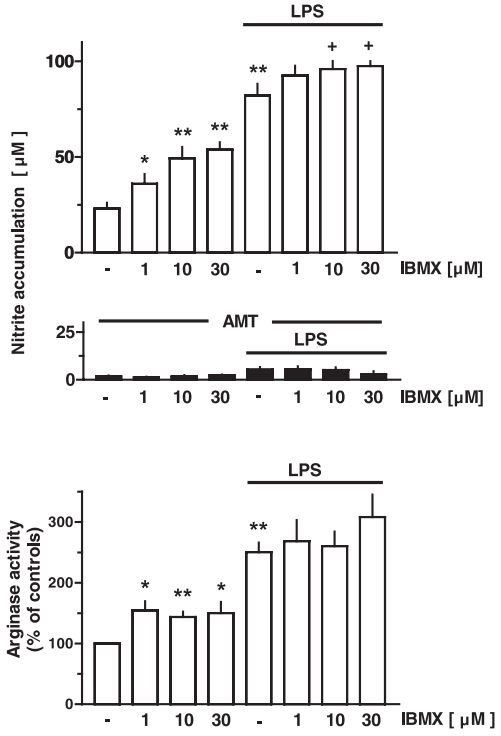

Fig. 1. Effects of lipopolysaccharides and IBMX on nitrite accumulation and arginase activity of alveolar macrophages. Cells (10^6/well) were cultured for 20 h under control conditions or in the presence of 1 μg/ml lipopolysaccharides and/or IBMX (at the concentrations given below the columns) and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT, 30 μM). The culture medium was collected and analysed for nitrite accumulation (upper part). Thereafter, the cells were lysed and the arginase activity was determined (lower part). Results are expressed in absolute terms (for nitrite accumulation, concentration in the culture medium in μM) or as percent of the mean value of the controls of the respective cell preparation (for arginase activity). Given are means ± S.E.M. of 6–12 experiments. $^*P<0.05$, $^{**}P<0.01$, when compared with the respective control value (absence of any test substance); $^+P<0.05$, when compared with lipopolysaccharides alone.

charides (1 μg/ml present during the culture period) caused a clear up-regulation of the arginase activity by 135% (Fig. 1). IBMX (up to 30 μM) caused an increase in arginase activity by only about 35% (Fig. 1).

Nitrite concentration detected in the culture media after 20-h culture under control conditions amounted to 23 ± 3 μM ($n = 20$) and the presence of 1 μg/ml lipopolysaccharides, a supramaximal effective concentration (Hammermann et al., 2000b), caused an increase to 82 ± 7 μM ($n = 12$). IBMX in a concentration-dependent manner also caused a clear increase in nitrite accumulation, to 53 ± 7 μM ($n = 12$), at the highest concentration of IBMX (30 μM) tested (Fig. 1). In the presence of 1 μg/ml lipopolysaccharides, IBMX caused still a further significant increase in nitrite accumulation (Fig. 1). As in previous experiments (Hammermann et al., 2001), AMT (30 μM), a specific inhibitor of iNOS (Nakane et al., 1995), suppressed almost completely the spontaneous and lipopolysaccharide-induced nitrite accumulation (Fig. 1). Moreover, AMT also prevented the IBMX-induced increase in nitrite accumulation (Fig. 1).

In order to test whether the cAMP–PKA pathway might be involved in the effect of IBMX, the effect of the PKA inhibitor K 5720 was studied. K 5720 (1 μM) caused a significant reduction in basal nitrite accumulation and a marked attenuation of IBMX induced increase in nitrite accumulation (Fig. 2).

3.2. Effects of lipopolysaccharides and IBMX on expression of iNOS and arginases I and II

As described in detail previously, iNOS mRNA was not detected in freshly isolated alveolar macrophages, but during culture under control conditions, there was a significant induction of iNOS mRNA, which peaked at about 5 h and thereafter, declined again (Hammermann et al., 2000b; Klasen et al., 2001). IBMX (30 μM) caused a small, but significant increase in iNOS mRNA, seen after

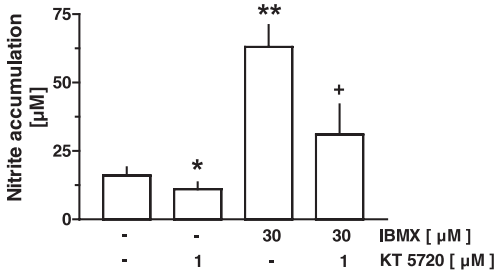

Fig. 2. Effects of the protein kinase A inhibitor KT 5720 and IBMX on nitrite accumulation by alveolar macrophages. Cells (10^6/well) were cultured for 20 h under control conditions or in the presence of KT 7720 and/or IBMX at the concentrations indicated. The culture medium was collected and analysed for nitrite accumulation. Results are expressed in absolute terms (nitrite concentration in the culture medium in μM). Given are means ± S.E.M. of 6–16 experiments. *$P < 0.05$, **$P < 0.01$, when compared with controls; $^{+}P < 0.05$, when compared with experiments with IBMX alone.

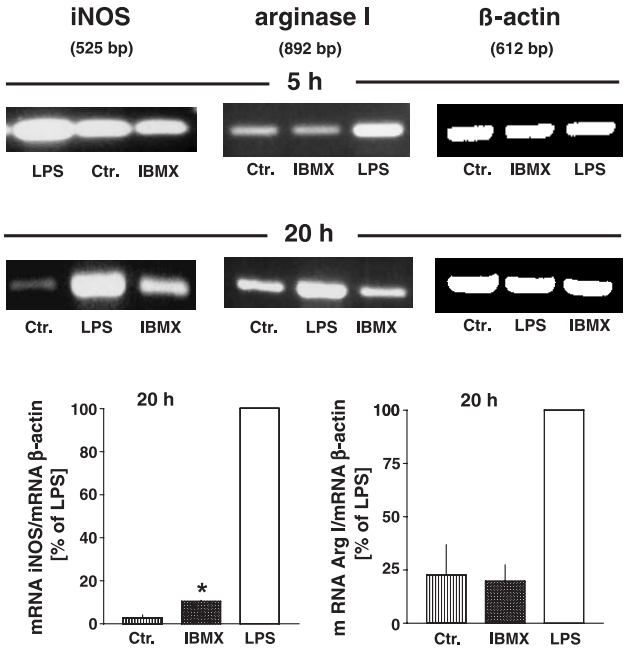

Fig. 3. Effects of lipopolysaccharides and IBMX on iNOS and arginase I mRNA in rat isolated alveolar macrophages. Cells (5×10^6/well) were cultured for 5 or 20 h in the absence or presence of lipopolysaccharides (1 μg/ml) or IBMX (30 μM). Total RNA was isolated and used for RT-PCR with specific primers for rat iNOS, arginase I. Upper part: Representative RT-PCRs showing one out of three to four similar experiments. The number of optimised PCR cycles was 25 for iNOS, arginase I and β-actin. Lower part: Quantification of the optical densities of the PCR bands of iNOS and arginase I after 20 h culture (ratio sample value/respective β-actin) expressed as percentage of the respective lipopolysaccharide values ± S.E.M. *$P < 0.05$, when compared with the controls (Ctr.).

20 h of exposure to IBMX (Fig. 3). The magnitude of this effect was markedly smaller than that caused by lipopolysaccharides. The time course of up-regulation of iNOS mRNA by IBMX was also markedly different from that observed by lipopolysaccharides, which caused a significant increase within 2 h and a maximal effect after 5 h (Hammermann et al., 2000b; Klasen et al., 2001; Fig. 3), whereas after 5 h of exposure to IBMX, an effect on iNOS mRNA was not yet detectable (Fig. 3). In contrast to iNOS mRNA, arginase I and II mRNA is constitutively expressed in rat alveolar macrophages. During 20-h culture under control conditions, arginase II mRNA declined significantly, whereas arginase I mRNA did not change significantly (Klasen et al., 2001). No significant effects of IBMX (30 μM) on the expression of arginase I or II mRNA were detected, neither after 5 nor 20 h of exposure (each $n = 3–4$; Fig. 3 and data not shown).

In contrast to the rather small increase in iNOS mRNA, protein levels were clearly enhanced after 20-h culture in the presence of IBMX, the effect reaching about 40% of that induced by lipopolysaccharides (Fig. 4). In freshly prepared alveolar macrophages (only 2-h adherence period) and in alveolar macrophages cultured for 5 h, both under control conditions or in the presence of IBMX, iNOS

protein levels were below the detection limit. In order to test whether IBMX might affect the lifetime of iNOS protein, iNOS was up-regulated by 20-h culture in the presence of lipopolysaccharides. Thereafter, protein de novo synthesis was inhibited by the addition of 30 μM cycloheximide and the cells were cultured for additional 20 h in the absence or presence of 30 μM IBMX. After inhibition of protein synthesis, IBMX did not affect the lipopolysaccharide-induced iNOS protein levels. The iNOS protein levels (ratio of the optical density iNOS/α-tubulin) in cells cultured after the lipopolysaccharides stimulus for 20 h in the presence of IBMX + cycloheximide amounted to 97.7 ± 4.8% ($n = 5$) of that detected in cells of the same cell preparation treated only with cycloheximide. Thus, the rate of degradation of iNOS protein appears not to be affected by IBMX.

3.3. Effects of isoenzyme selective phosphodiesterase inhibitors on nitrite accumulation

Finally, by the use of isoenzyme selective phosphodiesterase inhibitors, the role of specific phosphodiesterase isoenzymes should be demonstrated. Both the phosphodiesterase 4 selective inhibitor rolipram as well as the phosphodiesterase 3 selective inhibitor siguadozan caused a significant increase in nitrite accumulation, rolipram being more effective than siguadozan (Fig. 5). However, the maximum effects caused by rolipram or siguadozan were significantly smaller than that caused by IBMX. On the other hand, the phosphodiesterase 3/4 inhibitor benzafendrine was almost as effective as IBMX. At the highest concentration (30 μM) tested, no significant difference between the nitrite accumulation induced by either IBMX or benzafendrine was detected (Fig. 5).

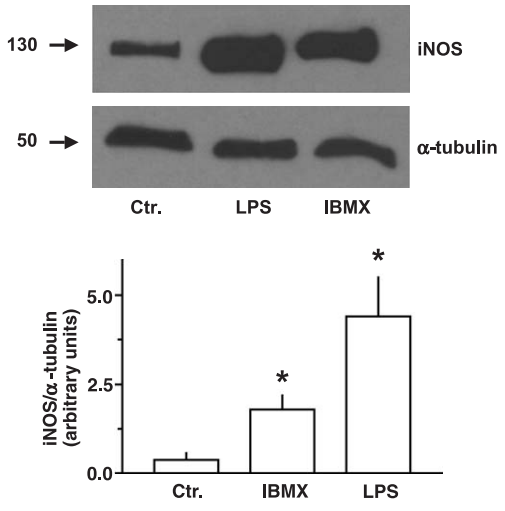

Fig. 4. Comparison of the effects of IBMX and lipopolysaccharides on iNOS protein levels in rat isolated alveolar macrophages. Cells (5×10^6/well) were cultured for 20 h in the absence or presence of lipopolysaccharides (1 μg/ml) or IBMX (30 μM). Thereafter, proteins were prepared and immunoblots for iNOS performed. Upper part: Representative examples of immunoblots. Lower part: Quantification of the optical densities of the protein bands (ratio sample value/respective α-tubulin) expressed as arbitrary units ± S.E.M. ($n = 4$). *$P < 0.05$, when compared with the controls (Ctr.).

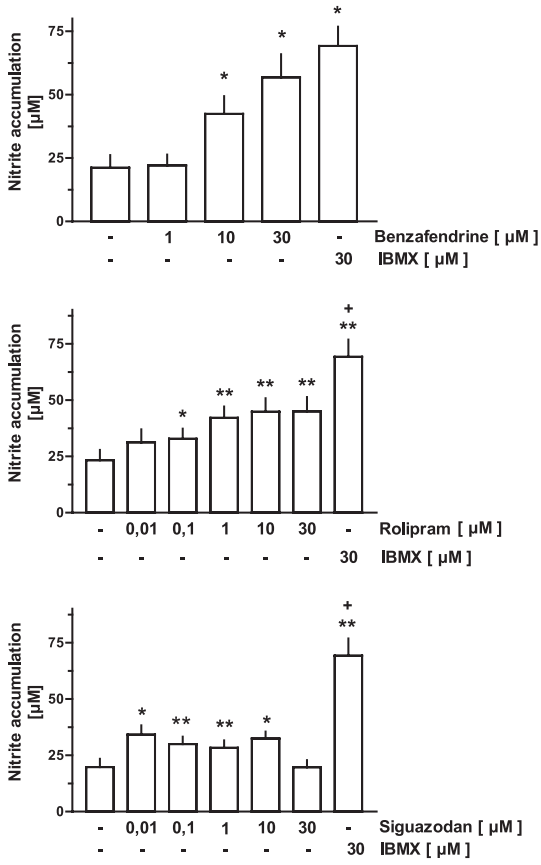

Fig. 5. Comparison of the effects of IBMX and the isoenzyme selective phosphodiesterase inhibitors benzafendrine (phosphodiesterase 3/4), rolipram (phosphodiesterase 4) and siguadozan (phosphodiesterase 3) on nitrite accumulation by alveolar macrophages. Cells (10^6/well) were cultured for 20 h under control conditions, in the presence of IBMX or one of the other phosphodiesterase inhibitors (at the concentration given below the columns). The culture medium was collected and analysed for nitrite accumulation. Results are expressed in absolute terms (nitrite concentration in the culture medium in μM). Given are means ± S.E.M. of 3–12 experiments. *$P < 0.05$, **$P < 0.01$, when compared with the controls (absence of any test substance); $^{+}P < 0.01$, when compared with the maximum effect of respective selective phosphodiesterase inhibitors.

4. Discussion

The present experiments revealed that inhibition of phosphodiesterases in rat alveolar macrophages in primary culture affected both L-arginine-dependent pathways; it caused a moderate up-regulation of arginase activity and an even stronger increase in nitrite accumulation. As nitrite accumulation was inhibited by the selective iNOS inhibitor AMT (Nakane et al., 1995), it is justified to use nitrite accumulation in the present cell preparation as a measure of iNOS-mediated NO synthesis.

An up-regulation of arginase by inhibition of phosphodiesterases has also been observed in rabbit alveolar macro-

phages. In that species, the effect of phosphodiesterase inhibition on arginase was substantially larger and no effect on NO synthesis was observed (Hammermann et al., 2000a). However, it should be noted that in rabbit alveolar macrophages, lipopolysaccharides failed also to induce any significant NO synthesis (Hey et al., 1995; Hammermann et al., 2000a). Since IBMX did not affect the mRNA levels of arginase I nor of arginase II, an increased transcriptional expression of either arginase enzyme may be excluded. Whether the increase in arginase activity after phosphodiesterase inhibition in rat alveolar macrophages was caused by direct modulation of the arginase enzyme or by an increase in the amount of enzyme (possibly by increased translation or delayed degradation) was not analysed in the present study because the overall effect was too small (35% increase).

However, the stimulatory effect on NO synthesis was studied in more detail. The magnitude of the effect of IBMX on nitrite accumulation (about 40% of the effect of a maximally effective lipopolysaccharide stimulus) correlated well with a corresponding increase in iNOS protein level (about 40% of the lipopolysaccharide stimulus). The observation that the specific PKA inhibitor KT 5270 (Kase et al., 1987) inhibited the IBMX-induced nitrite accumulation indicates that accumulation of cAMP and activation of the downstream PKA signalling mediated the up-regulation in NO synthesis. In other words, phosphodiesterases by inactivating cAMP appear to play a role as inhibitory control mechanism, limiting the up-regulation of NO synthesis in rat alveolar macrophages. A role of the cAMP–PKA pathway as mediator of stimulatory effects on iNOS expression and iNOS-mediated NO synthesis has been observed in rat alveolar macrophages (Greenberg et al., 1997) and murine RAW 264.7 macrophages (Chen et al., 1999) and different other cell types (e.g. Eberhardt et al., 1998; Cavicchi and Whittle, 1999; Kim et al., 2000; Samardzic et al., 2000). In contrast to the observations by Chen et al. (1999), Morris et al. (1998) failed to demonstrate any stimulatory effect of 8-bromo-cAMP on iNOS mRNA expression and nitrite accumulation in RAW 264.7 macrophages, but observed an inhibitory effect on interferon-γ-induced iNOS expression. Likewise, in Kupffer cells (Mustafa and Olson, 1998) and murine primary fibroblasts (Samardzic et al., 2000), cAMP mediated inhibitory effects on iNOS expression. Thus, the effect of the cAMP–PKA pathway on iNOS expression appears to be cell type-specific and may even vary in the same cell type, possibly depending on the functional state of the respective cell and in the presence of other modulators.

In the present experiments, the IBMX-induced increase in nitrite accumulation correlated with an increase in iNOS protein levels and the magnitude of both effects (about 40% of the effect of lipopolysaccharides) agreed well. Although the present RT-PCR technique does not allow to measure absolute levels of mRNA, it is evident that lipopolysaccharides caused a much stronger increase in iNOS mRNA than IBMX. Moreover, in contrast to the effect of lipopolysaccharides which is seen quite rapidly (within 2 h, Hammermann et al., 2000b; Klasen et al., 2001; see also Fig. 2), the IBMX-induced increase in iNOS mRNA occurred with some delay, it was seen after 20 h, but not yet after 5-h exposure to IBMX (Fig. 2). In rat mesangial cells, it was demonstrated that cAMP caused the up-regulation of several transcription factors known to be a target for PKA such as cAMP-responsive element binding protein (CREB) (Gonzalez and Montminy, 1989) and several members of the C/enhancer-binding protein (CCAAT/EBP) family (Metz and Ziff, 1991). Further, cAMP promoted the binding of these transcription factors to the nuclear DNA, finally resulting in an induction of the iNOS promoter (Eberhardt et al., 1998). The necessity first to induce the downstream target proteins of the cAMP–PKA pathway mediating the activation of iNOS transcription could explain the delayed response.

On the other hand, the overall rather small increase in iNOS mRNA in comparison to the relative large increase in iNOS protein suggests that the IBMX-induced up-regulation of iNOS may not only be the result of a transcriptional activation of iNOS expression. It has been shown that degradation of iNOS occurs via proteasomal proteolysis after ubiquitination (Kolodziejski et al., 2002). It appears possible that this process is regulated via the cAMP–PKA pathway. However, the lack of effect of IBMX on iNOS protein levels after inhibition of protein synthesis by cycloheximide excludes that phosphodiesterase inhibition may cause a retarded degradation of the iNOS protein at least as direct effect. An induction of another protein which might affect the stability of the iNOS protein cannot be excluded by the present observations. Finally, the possibility remains that activation of the cAMP–PKA pathway may enhance the translational effectiveness of the iNOS protein synthesis.

As already mentioned in Introduction, there are at least 11 phosphodiesterase families, and as demonstrated in different species, alveolar macrophages express several phosphodiesterase isoenzymes, phosphodiesterase 1, phosphodiesterase 3 and phosphodiesterase 4 representing the major activities (see Torphy, 1998; Schudt et al., 1999). At present, the phosphodiesterases 1–5 can well be discriminated by pharmacological tools. Therefore, by the use of subtype selective inhibitors, the role of specific phosphodiesterase isoenzymes in the control of iNOS-mediated NO synthesis in alveolar macrophages was characterized. The phosphodiesterase 3/4 inhibitor benzafendrine caused an increase in nitrite accumulation similar to IBMX, whereas the phosphodiesterase 4 inhibitor rolipram was significantly less effective than IBMX, but somewhat more effective than the phosphodiesterase 3 inhibitor siguadozan. Thus, these experiments indicate that both phosphodiesterase 4 and, to a smaller extent, phosphodiesterase 3 are involved in the control of iNOS-mediated NO synthesis in rat alveolar macrophages. Since both phosphodiesterase 3 and phosphodiesterase 4 show substrate specificity for cAMP, these

observations, too, indicate that a rise in cAMP is responsible for the up-regulation of iNOS in alveolar macrophages. Phosphodiesterase 3 and, particularly, phosphodiesterase 4 have also been shown to be involved in the control of bronchoconstriction and inflammatory response in the airways (e.g. Nicholson et al., 1995; Spina et al., 1995; Ortiz et al., 1996; for review, see Torphy, 1998; Schmidt et al., 1999; Schudt et al., 1999; Torphy and Page, 2000). Whether the observed effects of phosphodiesterase inhibitors on L-arginine metabolism in rat alveolar macrophages may contribute to beneficial or unwanted effects of phosphodiesterase inhibitors used in treatment of obstructive and inflammatory airway diseases remains to be determined. In particular, the moderate increase in NO release after phosphodiesterase inhibition could contribute to the anti-inflammatory effects of phosphodiesterase inhibitors, as NO can inhibit the release of several pro-inflammatory mediators and cytokines (e.g. Brunn et al., 1997; Tsao et al., 1997; Villarete and Remick, 1997).

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