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Inhibition of nitric oxide synthase abrogates lipopolysaccharides-induced up-regulation of L-arginine uptake in rat alveolar macrophages

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1 It was tested whether the inducible nitric oxide synthase (iNOS) pathway might be involved in lipopolysaccharides-(LPS)-induced up-regulation of L-arginine transport in rat alveolar macrophages (AMΦ).

2 AMΦ were cultured in absence or presence of LPS. Nitrite accumulation was determined in culture media and cells were used to study [^{3}H]-L-arginine uptake or to isolate RNA for RT–PCR.

3 Culture in presence of LPS (1 μg ml^{-1}, 20 h) caused 11 fold increase of nitrite accumulation and 2.5 fold increase of [^{3}H]-L-arginine uptake.

4 The inducible NO synthase (iNOS) inhibitor 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) present alone during culture had only marginal effects on [^{3}H]-L-arginine uptake. However, AMT present during culture additionally to LPS, suppressed LPS-induced nitrite accumulation and LPS-stimulated [^{3}H]-L-arginine uptake in the same concentration-dependent manner. AMT present only for the last 30 min of the culture period had similar effects on [^{3}H]-L-arginine uptake. AMT present only during the uptake period also inhibited LPS-stimulated [^{3}H]-L-arginine uptake, but with lower potency.

5 The inhibitory effect of AMT could not be opposed by the NO releasing compound DETA NONOate.

6 LPS caused an up-regulation of the mRNA for the cationic amino acid transporter CAT-2B, and this effect was not affected by AMT.

7 AMT (100 μM) did not affect L-arginine transport studied by electrophysiological techniques in *Xenopus laevis* oocytes expressing either the human cationic amino acid transporter hCAT-1 or hCAT-2B.

8 In conclusion, iNOS inhibition in rat AMΦ abolished LPS-activated L-arginine uptake. This effect appears to be caused by reduced flow of L-arginine through the iNOS pathway.

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Keywords: Alveolar macrophages; nitric oxide synthase; L-arginine transport; cationic amino acid transporter; CAT-2B

Abbreviations: AMΦ, alveolar macrophages; AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine; CAT, cationic amino acid transporter; EITU, S-ethyl-isothiourea; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine monoacetate; LPS, lipopolysaccharides; MΦ, macrophages; NO, nitric oxide

Introduction

In macrophages (MΦ), including alveolar MΦ (AMΦ) synthesis and release of nitric oxide (NO) is part of the effector mechanisms in the non-specific defence system (see Moncada *et al*., 1991), but NO also plays a role as signalling molecule to control inflammatory reactions (e.g. Brunn *et al*., 1997; Thomassen *et al*., 1997). Three isoenzymes of NO synthase (NOS) have been identified (see Moncada *et al*., 1991; Förstermann & Kleinert, 1995), and the inducible form, iNOS (also named NOS II), is responsible for the NO production in MΦ and AMΦ (e.g. Stuehr *et al*., 1991; Yui *et al*., 1991; Xie *et al*., 1992; Hey *et al*., 1995). Once expressed, iNOS is active and the amount of NO synthesized by MΦ depends critically on the availability of L-arginine (e.g. Jorens *et al*., 1991; Baydoun *et al*., 1994; Hammermann *et al*., 1998; Closs *et al*., 2000; Messeri Dreißig *et al*., 2000). It has been observed in different types of MΦ that inductors of iNOS such as bacterial lipopolysaccharides (LPS) or the cytokine interferon-γ (IFN-γ) caused an up-regulation of L-arginine uptake (e.g. Bogle *et al*., 1992; Sato *et al*., 1992; Hammermann *et al*., 1999; 2000; Messeri Dreißig *et al*., 2000).

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 $B^{0,+}$) (see Closs, 1996; Devés & Boyd, 1998). In MΦ system y^+ plays a particular role and appears to mediate the LPS-stimulated L-arginine transport (Bogle *et al*., 1992; Messeri Dreißig *et al*., 2000). At the

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molecular level the transport properties of system y^+ could be ascribed to a family of cationic amino acid transporters (CATs) (see Closs, 1996; Devés & Boyd, 1998). In rat AMΦ mRNA of CAT-1 and of a splicing variant of the CAT-2 gene (CAT-2B) were found to be expressed (Racké *et al*., 1998). The LPS-induced up-regulation of L-arginine transport was accompanied by an increased expression of mRNA for CAT-2B, but not for CAT-1 (Messeri Dreißig *et al*., 2000).

The question arose whether the iNOS pathway might play a role in the up-regulation of L-arginine transport and CAT-2B expression which occurred in association with the induction of iNOS. Therefore, the effect of inhibition of iNOS on the LPS-induced up-regulation of L-arginine transport and the expression of CAT-2B was studied in rat AMΦ, as a model of primary MΦ.

Methods

Preparation and culture of AMΦ

Sprague Dawley rats (own breeding) of either sex were killed by stunning followed by exsanguination. Lung and trachea were excised en bloc and lavaged thrice by instilling 10–15 ml of cold phosphate buffered saline (see Holt, 1979; Hey *et al*., 1995). Usually, for one preparation of AMΦ, lavage fluids from 4–6 lungs were pooled and centrifuged at $400 \times g$ for 10 min. Thereafter, cells were resuspended in DME/F-12 medium supplemented with 5% foetal calf serum, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 μg ml^{-1} amphotericin B and plated in 12-well plates (10^6 AMΦ for uptake and nitrite accumulation studies), or $4*10^6$ AMΦ were disseminated on 35 mm culture dishes (for RT–PCR). AMΦ were allowed to adhere for 2 h (37°C; 5% CO_2), before the medium was renewed to remove non-adherent cells. The adherent cells consisted of more than 95% AMΦ according to morphological criteria (May Grünwald-Giemsa staining as described by Rick (1974)). Thereafter, AMΦ were cultured for 5 or 20 h in the absence or presence of test substances.

[^{3}H]-L-arginine uptake studies

At the end of the 20 h culture period medium was removed and kept at −80°C until used for nitrite determination (see below). AMΦ were subsequently incubated at 37°C for 2 min in 0.5 ml [^{3}H]-L-arginine (37 kBq, 0.1 μM) containing Krebs-HEPES solution (composition [mM]: NaCl 120; KCl 5.4; $CaCl_2$ 1.25; $MgCl_2$ 1.2; Na_2EDTA 0.03; L-(+)-ascorbic acid 0.06; HEPES 20.0; (adjusted to pH 7.4 using NaOH) and D-(+)-glucose 10). The 2 min incubation period was chosen as standard incubation protocol because [^{3}H]-L-arginine uptake was linear between 1 and 3 min (Hey *et al*., 1997; Racké *et al*., 1998). In some experiments [^{3}H]-L-arginine concentration was enhanced to 100 μM or 1 mM, respectively. In one series of experiments the time of incubation with [^{3}H]-L-arginine was shortened to 1 min. Thereafter, cells were lysed in 0.5 ml Tris/Triton (0.1% v v^{-1}) solution followed by determination of the cellular radioactivity and protein content using the DC Protein Assay (BioRad). [^{3}H]-L-arginine uptake was expressed either in absolute terms (pmol mg $protein^{-1}$) or as per cent of the uptake observed in respective controls of the respective cell preparation.

Radioactivity in the cell extracts was measured by liquid scintillation spectrometry in a Tri-Carb 2100 TR (Canberra Packard, Dreieich, Germany). External standardization was used to correct for counting efficiency.

Nitrite assay

As a measure of NO synthesis during the culture period nitrite which accumulated in the culture media was determined by a spectrophotometric assay based on the Griess reaction as described previously (Hey *et al*., 1995). Briefly, 400 μl Griess reagent (sulfanilic acid 1%, N(1-naphthyl)ethylenediamine hydrochloride 0.1% dissolved in 2.5% (w v^{-1}) H_3PO_4) were 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 (nmol (10^6 $cells)^{-1}$) or per cent of the controls of the respective cell preparation.

Extraction of RNA and Reverse Transcription Polymerase Chain Reaction (RT–PCR)

Total RNA was isolated from AMΦ cultured for 5 h in the absence or presence of LPS and other test substances using Trizol® reagent. This time period was chosen because the LPS-mediated induction of iNOS and CAT-2B was observed to be maximal after 5 h exposure to LPS (Messeri Dreißig *et al*., 2000). The first strand cDNA was synthesized from 2 μg total RNA using $Oligo(dT)_{18}$ primer and AMV 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 EMBL sequences for rat (r) β-actin (accession number V01217; J00691), riNOS (L12562), rCAT-1 (L10151), and rCAT-2B (U53927). Primer pairs were: 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, Holland) 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, Ebersberg, Germany).

Preparation of cRNA for microinjection

The plasmids pSPhCAT-1AB1 and phCAT-2B 181 were linearized with *Sal*I and then transcribed *in vitro* from the

SP6 promoter of pSP64T as described previously (Closs *et al*., 1997).

Preparation of Xenopus laevis *oocytes*

Bags of the ovary of the African clawed frog *Xenopus laevis* (anaesthetized with ice and 0.1% 3-aminobenzoic acid ethyl ester) were surgically removed and placed in nominally Ca^{2+}-free saline solution (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, HEPES 10; pH 7.6). Oocytes were either mechanically singled out, using a platinum loop, or obtained by enzymatic treatment to remove their follicular envelopes (collagenase A, 1.9 units 10 ml^{-1}, 30–40 min), and stored in a modified Leibovitz medium at 4°C up to 7 days. The Leibovitz medium was modified by dilution with H_2O (1 : 2) and the addition of 1 mM L-glutamine, 100 μg ml^{-1} gentamycine and 15 mM HEPES (pH 7.6). The cationic amino acid content of the medium amounted to 1.45 mM L-arginine and 0.25 mM L-lysine. cRNA (25 ng (25 nl $H_2O)^{-1}$) was injected into the oocytes under microscopic control using a pneumatic transjector (5246, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and a micromanipulator (MMJ, Märzhäuser, Wetzlar, Germany). The cRNA-injected oocytes were then incubated at 18°C for 48–72 h in the modified Leibovitz medium for translation, processing and embedding of the mature proteins into the cell membrane, before the electrophysiological experiments were carried out. H_2O-injected or native oocytes were used as controls.

Measurement of membrane potential

Single oocytes were placed on a plastic grid in an organ bath of about 1 ml which was built into a Perspex® block that also contained a main reservoir of 100 ml modified Ringer solution (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5; pH was adjusted to 7.5 by NaOH), kept at 20°C. Communication between the two compartments was provided by connecting bores through which the fluids were driven by gas pressure (O_2). Thereby, substances could be easily added to or removed from the main reservoir by a rapid fluid exchange, without mechanically or electrically disturbing the test compartment. The membrane potential (E_M) of oocytes was recorded intracellularly with conventional micropipettes made from borosilicate glass (Science Products, Frankfurt, Germany), filled with 3 M KCl (resistances between 10 and 20 MΩ) which were connected to a unity gain buffer amplifier (current clamp). The oocytes were impaled with the help of a micromanipulator under microscopic control, and the recorded signals were displayed on scope Nicolet 310 (Nicolet Instr. Co., Madison, WI, U.S.A.) and stored digitally on floppy disks in a DOS-compatible format.

Measurement of membrane currents

In the same experimental set-up as described above, two electrodes whose tips were broken down to larger diameters, yielding resistances between 0.8 and 1.2 MΩ, were inserted into the same oocyte. The two-electrode voltage-clamp was performed as described (Stühmer, 1992). All measurements were carried out in conjunction with a TEC05 two-electrode voltage-clamp amplifier (NPI Electronics, Tamm, Germany). Data were stored on a hard disk of a pentium-processor based DOS-compatible microcomputer, running pClamp 5.7 software (Axon Instruments Inc., Foster City, CA, U.S.A.), in conjunction with an AD-DA converter (DigiData 1200, Axon Instruments Inc.) which was also used for the generation of voltage commands and data analysis. Experiments were excluded from analysis when leak currents increased >10%, indicating membrane damage at the sites of impalements. Due to the lack of fast changes in membrane ion conductances, in response to any voltage clamp jumps, the currents were not electronically compensated for membrane capacity or resistance.

Statistical analysis

All values are means ± s.e.mean of n experiments. EC_{50} values were calculated with the help of a computer programme (Tallarida & Murray, 1988). Statistical significance of differences was evaluated by Student's t-test. When multiple comparisons were performed the significance of differences was evaluated by ANOVA followed by the modified t-test according to Dunnett or Bonferroni when appropriate using the computer programme GraphPad InStat (GraphPad Software, San Diego, U.S.A.). $P<0.05$ was accepted as significant.

Drugs and materials

L-[2,3-^{3}H]-arginine HCl (1481 GBq $mmol^{-1}$, Dupont, Dreieich, Germany); Amphotericin B, Dulbecco's modification of Eagle's/Ham's F-12 medium (DME/F-12 medium), lipopolysaccharides from *Escherichia coli* 0127:B8, RedTaq DNA polymerase (all Sigma, München, Germany); 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT), DETA NO-NOate, S-ethyl-isothiourea (EITU) and N^G-monomethyl-L-arginine monoacetate (L-NMMA) (Alexis Deutschland, Grünberg, Germany); collagenase A (Boehringer Mannheim, Germany); foetal calf serum (Vitromex, Germany). DC Protein Assay (BioRad, Munich, Germany), Trizol® reagent for RNA isolation (Life Technologies, Karlsruhe, Germany); AMV reverse transcriptase (Promega, Mannheim, Germany). All desoxynucleotides for RT–PCR were obtained from MWG Biotech (Ebersberg, Germany).

Results

*AMT a potent inhibitor of iNOS in AM*Φ

After 20 h culture of AMΦ in the absence or presence of 1 μg ml^{-1} LPS, the amounts of nitrite detected in the culture media were 6.3 ± 0.6 (n = 39) and 57.4 ± 4.5 (n = 30) nmol (10^6 cells*20 h)$^{-1}$, respectively. Presence of AMT, in addition to LPS, caused a concentration-dependent reduction of the nitrite accumulation, by 97% at 100 μM, IC_{50}: 2 μM (Figure 1). EITU or L-NMMA (each 100 μM) present together with LPS caused a reduction of the nitrite accumulation by 88.4 ± 1.4% (n = 9) and 65.9 ± 2.2% (n = 12). As AMT appeared to be the most effective iNOS inhibitor in rat AMΦ it was used in most of the following experiments. Basal nitrite accumulation was also reduced by AMT, by 68.8 ± 1.6% (n = 8) and 92.0 ± 1.7% (n = 11) at 1 and 100 μM AMT, respectively (and data not shown).

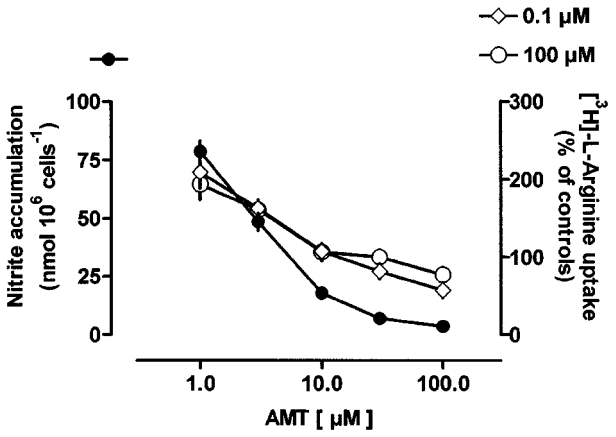

Figure 1 Concentration-dependent effects of AMT on nitrite accumulation and [^{3}H]-L-arginine uptake in LPS-stimulated rat AMΦ. Isolated AMΦ (10^6 cells per well) were cultured for 20 h in the absence or presence of LPS (1 μg ml $^{-1}$) and AMT (at the concentrations indicated by the abscissa). Thereafter, the culture medium was collected for the determination of nitrite and the cells incubated for 2 min in 0.5 ml Krebs-HEPES solution containing [^{3}H]-L-arginine (37 kBq, 0.1 or 100 μM) followed by determination of the cellular radioactivity. Ordinates: Nitrite accumulation expressed in absolute terms and [^{3}H]-L-arginine uptake expressed as per cent of the respective mean value observed in controls (absence of LPS) of the individual cell preparation. Values in the absence of AMT were 93.6 ± 5.4 nmol (10^6 cells)$^{-1}$ for nitrite and $267 \pm 13\%$ and $377 \pm 33\%$ for 0.1 and 100 μM [^{3}H]-L-arginine, respectively. Results show means $\pm$ s.e.mean of 8–12 experiments.

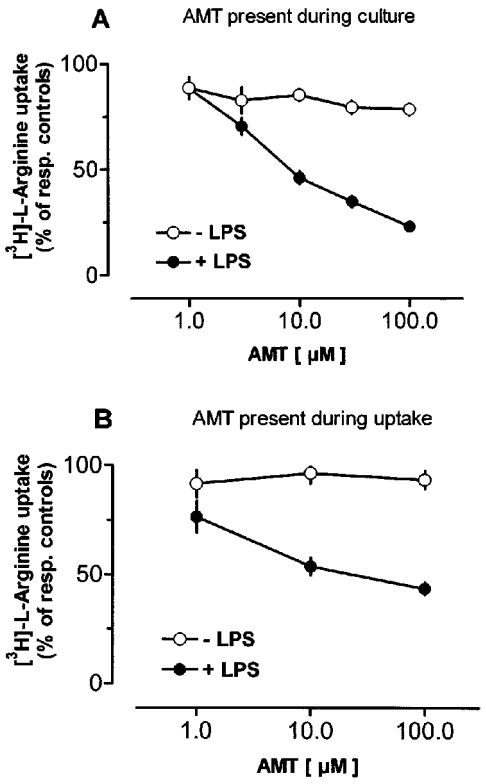

Figure 2 Concentration-dependent effects of AMT on [^{3}H]-L-arginine uptake in rat AMΦ, cultured for 20 h in the absence or presence of 1 μg ml^{-1} LPS. After the culture period the cells were incubated for 2 min in 0.5 ml Krebs-HEPES solution containing [^{3}H]-L-arginine (37 kBq, 0.1 μM) followed by determination of the cellular radioactivity. AMT at the concentrations indicated by the abscissae was present either during the culture period (A) or only during the uptake period (B). Ordinates: [^{3}H]-L-arginine uptake expressed as per cent of the respective mean value observed in controls of the individual cell preparation. Results show means $\pm$ s.e.mean of 8–12 experiments.

Effects of iNOS inhibition on L-arginine transport in AMΦ

After 20 h culture under control conditions, rat AMΦ incubated for 2 min with [^{3}H]-L-arginine (37 kBq ml^{-1}, 0.1 μM) accumulated 6.8 ± 0.8 pmol L-arginine mg protein^{-1} ($n = 25$). After culture in the presence of 1 μg ml^{-1} LPS, [^{3}H]-L-arginine uptake was increased to 16.5 ± 1.72 pmol L-arginine mg protein^{-1} ($n = 25$). Presence of AMT alone (up to 100 μM) in the culture medium caused only a minor reduction (20%) of the subsequently studied [^{3}H]-L-arginine uptake (Figure 2A). However, when AMT was present together with LPS, it caused in a concentration-dependent manner a reduction of the subsequently studied [^{3}H]-L-arginine uptake, by 77% at 100 μM (Figures 1 and 2A). In other words, presence of AMT prevented the LPS-induced activation of [^{3}H]-L-arginine uptake with an IC_{50} of 2 μM, but had only marginal effects on basal [^{3}H]-L-arginine transport (Figure 2A). Likewise, 100 μM EITU present alone during the culture period had no effect on [^{3}H]-L-arginine uptake ($93 \pm 7\%$ of controls, $n = 8$), but inhibited [^{3}H]-L-arginine uptake by $55 \pm 4\%$ ($n = 8$) when present in combination with LPS, i.e. EITU also attenuated largely the LPS-induced up-regulation of [^{3}H]-L-arginine transport.

Similar inhibitory effects of AMT were observed when the concentration of [^{3}H]-L-arginine in the uptake medium was enhanced to 100 μM. Rat AMΦ incubated for 2 min with 100 μM [^{3}H]-L-arginine (37 kBq ml^{-1}) accumulated 3.0 ± 0.3 and 10.6 ± 0.6 nmol L-arginine mg protein $^{-1}$ after culture in the absence or presence of 1 μg ml^{-1} LPS, respectively (each $n = 12$). Presence of AMT alone (up to 100 μM) in the culture medium caused only a minor reduction (20%) of the subsequently studied [^{3}H]-L-arginine uptake (Figure 3 and data not shown), but AMT present together with LPS, suppressed the LPS-stimulated [^{3}H]-L-arginine uptake in a concentration-dependent manner (Figure 1). Finally, when the concentration of [^{3}H]-L-arginine in the uptake medium was enhanced to 1 mM, the inhibitory effect of AMT remained unchanged (Figure 3). Rat AMΦ incubated for 2 min with 1 mM [^{3}H]-L-arginine (37 kBq ml^{-1}) accumulated 8.1 ± 0.9 and 19.8 ± 1.2 nmol L-arginine mg protein^{-1} after culture in the absence or presence of 1 μg ml^{-1} LPS, respectively (each $n = 8$). After culture in the presence of AMT the LPS-stimulated [^{3}H]-L-arginine uptake was prevented, but [^{3}H]-L-arginine uptake in cells not exposed to LPS was not significantly affected (Figure 3).

When present only during the 2 min uptake period, AMT up to 100 μM failed to affect [^{3}H]-L-arginine uptake in control AMΦ, but caused in concentration-dependent manner a reduction of [^{3}H]-L-arginine uptake in LPS-stimulated AMΦ (Figure 2B). However, it appeared that the inhibition produced by AMT in these experiments was somewhat less pronounced compared with that observed in experiments in which the drug had been present during the culture period.

In order to get more information about the site of action of AMT two additional sets of experiments were performed. When AMT was only present during the last 30 min of the culture period, i.e. the cells were acutely loaded with the NOS inhibitor, the LPS-activated [^{3}H]-L-arginine uptake was inhibited (Figure 4) like in the experiments in which AMT had been present during the whole culture period (Figure 2A). On the other hand, when AMT was only present during the uptake period and the uptake period was shortened from usually 2 to 1 min, the inhibitory effect of AMT was significantly attenuated (Figure 4).

In an additional series of experiments it was tested whether the NO releasing compound DETA NONOate could oppose the inhibitory effect of AMT. AMΦ were cultured in the presence of LPS for 20 h and AMT (30 μM) was present during the last 30 min of the culture period. DETA NONOate (100, 300 or 1000 μM) present either together with AMT (i.e. for the last 30 min of culture, each $n=3$), or present only during the 2 min uptake period (each $n=3$) or present during the last 30 min of the culture period plus the 2 min uptake period (each $n=6$), did not reverse the AMT-induced reduction of [^{3}H]-L-arginine uptake. In contrast DETA NONOate (100 and 300 μM) caused a further reduction of the [^{3}H]-L-arginine uptake by about 40% under all conditions studied, and this effect was lost at the higher concentration of 1000 μM DETA NONOate (data not shown).

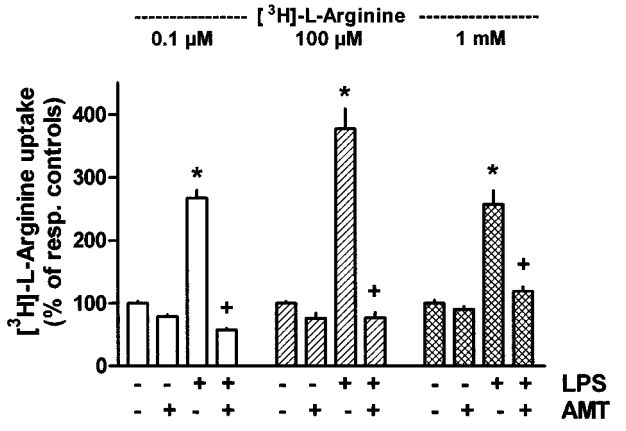

Figure 3 Effects of AMT on the uptake of [^{3}H]-L-arginine in different concentrations in rat AMΦ. Isolated AMΦ (10^6 cells per well) were cultured for 20 h in the absence or presence of LPS (1 μg ml^{-1}) and AMT (100 μM). Thereafter cells were incubated for 2 min in 0.5 ml Krebs-HEPES solution containing [^{3}H]-L-arginine (37 kBq, 0.1, 100 μM or 1 mM) followed by determination of the cellular radioactivity. Height of columns: [^{3}H]-L-arginine uptake expressed as per cent of the respective mean value observed in controls (absence of LPS) of the individual cell preparation (for absolute values see text). Results show means + s.e.mean of 8–12 experiments. Significance of differences: $*P<0.01$ when compared with the respective control value; $+P<0.01$ when compared with respective value in the absence of AMT.

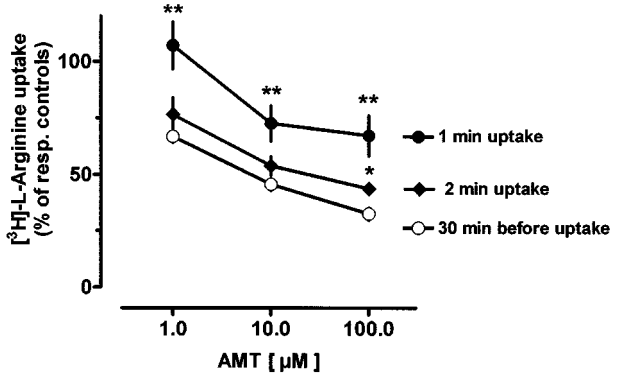

Figure 4 Concentration- and time-dependent effects of AMT on [^{3}H]-L-arginine uptake in rat AMΦ, cultured for 20 h in the presence of 1 μg ml^{-1} LPS. After the culture period the cells were incubated for 2 min or 1 min in 0.5 ml Krebs-HEPES solution containing [^{3}H]-L-arginine (37 kBq, 0.1 μM) followed by determination of the cellular radioactivity. AMT at the concentrations indicated by the abscissa was present either only during the last 30 min of the culture period or only during the 1 or 2 min uptake period. In controls, the 1 min uptake amounted to 60 ± 6% ($n=11$) of the 2 min values of the respective cell preparation. Ordinate: [^{3}H]-L-arginine uptake expressed as per cent of the respective mean value observed in controls of the individual cell preparation. Results show means ± s.e.mean of 5–11 experiments. $*P<0.05$ and $**P<0.01$ compared with the respective value '30 min before uptake'.

Effects of iNOS inhibition on CAT-2B expression in AMΦ

It was also tested whether inhibition of NO synthesis might affect the mRNA expression of cationic amino acid transporters. In previous experiments it was observed that LPS caused a parallel up-regulation of mRNA for iNOS and CAT-2B, and that the maximal effect was seen after 5 h exposure to LPS (Messeri Dreißig *et al*., 2000; Hammermann *et al*., 2000). In the present experiments, the up-regulation of the mRNA for CAT-2B seen after 5 h exposure to LPS was not affected when the NO synthesis had been inhibited by 30 μM AMT (Figure 5) or 100 μM EITU (data not shown). Likewise, the LPS-induction of iNOS mRNA was not affected by the NOS inhibitors (data not shown). Moreover, mRNA of CAT-1 was not affected neither by LPS alone nor in combination with the NOS inhibitors (data not shown).

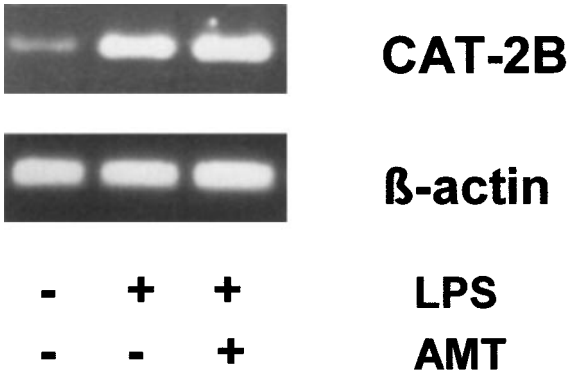

Figure 5 Effects of LPS in the absence or presence of AMT (30 μM) on the expression of mRNA for CAT-2B in rat AMΦ. AMΦ were cultured for 5 h in the absence or presence of 1 μg ml^{-1} LPS and additional presence of 30 μM AMT as indicated. Thereafter total RNA was isolated and RT–PCR (25 cycles) was performed using primer pairs specific for rat CAT-2B and β-actin. Shown is one out of three similar experiments.

Effects of AMT on CATs expressed in oocytes

The membrane potential (E_M) of both untreated and H_2O-injected oocytes was around −50 mV indicating that the injection procedure did not alter significantly the membrane properties of native oocytes (not shown, see Nawrath *et al*., 2000). In contrast, oocytes injected with cRNA coding for hCAT-2B were found to develop more negative membrane potentials in L-arginine-free solution, when loaded before with cationic amino acids, as described previously (Nawrath *et al*., 2000). When control oocytes were exposed to 1 mM L-arginine outside, E_M remained more or less unchanged, although minor depolarizations of 1–2 mV were occasionally observed (not shown, see Nawrath *et al*., 2000). In contrast, oocytes expressing hCAT-1 or hCAT-2B were depolarized in the presence of 1 mM L-arginine outside (Figure 6). AMT 100 μM did not change the resting potentials of either H_2O- (not shown) or cRNA-injected oocytes (Figure 6). AMT 100 μM did not alter the effects of 1 mM L-arginine on E_M in all preparations tested (Figure 6). The L-arginine-induced depolarization was concentration-dependent (not shown) and completely reversible after washout of the amino acid by repeated exchange of the bath solution. Therefore, the effects of L-arginine and AMT on L-arginine-induced changes in E_M could be tested in the same oocyte.

In further experiments, E_M was held constant at −60 mV, and the membrane current was observed under control conditions and in the presence of L-arginine outside. Due to the relatively large E_M (more negative than −60 mV) in hCAT-2B-injected oocytes, a positive holding current was observed at the command voltage of −60 mV (Figure 7). The addition of 1 mM L-arginine produced a large inward current. The effect of L-arginine on membrane current was completely reversible after washout. AMT (100 μM) was then added to the bath. Under these conditions, the repeated exposure to L-arginine (1 mM) produced virtually the same inward current, as seen during the first exposure. Figure 8 summarizes the lack of effects of AMT (100 μM) in both hCAT-2B and hCAT-1-injected oocytes on inward currents in response to L-arginine (1 mM).

Discussion

Induction of iNOS in rat AMΦ by LPS is accompanied by a marked up-regulation of L-arginine transport (Hammermann *et al*., 1999; Messeri Dreißig *et al*., 2000), and the present experiments showed in addition that this LPS-stimulated L-arginine uptake was abrogated in cells treated with NOS inhibitors, either AMT, a potent and selective inhibitor of iNOS (Nakane *et al*., 1995) or EITU, a potent inhibitor of all isoforms of NOS (Southan *et al*., 1995). In contrast, basal L-arginine transport was only marginally affected by treatment with these NOS inhibitors.

As outlined in the Introduction rat AMΦ express at least two different cationic amino acid transporters, CAT-1 and CAT-2B (Racké *et al*., 1998), and LPS-mediated activation of

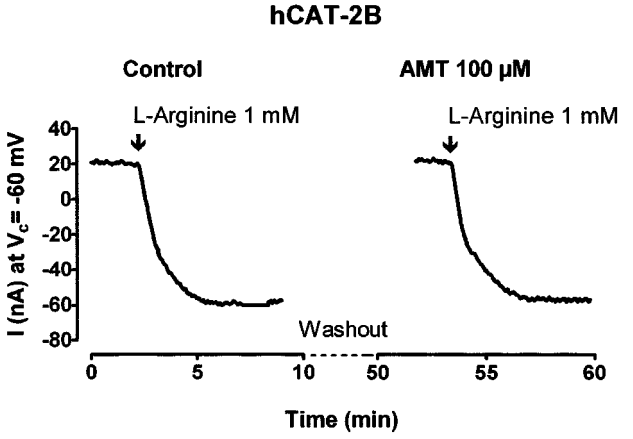

Figure 7 Membrane current in a *Xenopus* oocyte expressing hCAT-2B, as influenced by L-arginine 1 mM and AMT 100 μM. Original record. All effects were observed sequentially in the same preparation. AMT did not alter the magnitude of L-arginine-induced currents.

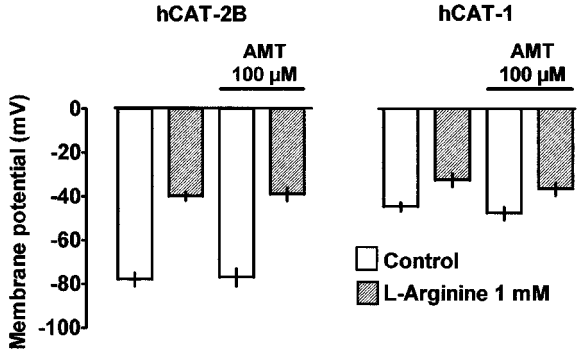

Figure 6 Membrane potentials in *Xenopus* oocytes expressing hCAT-2B or hCAT-1, as influenced by L-arginine 1 mM and AMT 100 μM. Shown are means±s.e.mean of three preparations each. In both groups of oocytes, the values were sequentially measured (1) in Ringer solution; (2) 5 min after the addition of L-arginine; (3) after repeated washout in Ringer solution and 10 min after the addition of AMT; and (4) 5 min after the addition of L-arginine in the presence of AMT. Note that the depolarizing effect of L-arginine was not influenced by AMT, neither in hCAT-2B nor hCAT-1 injected oocytes.

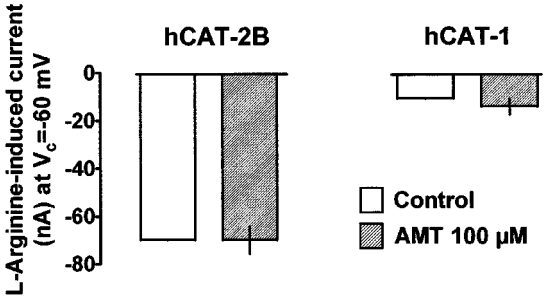

Figure 8 L-arginine-induced membrane currents in *Xenopus* oocytes expressing hCAT-2B or hCAT-1, as influenced by AMT 100 μM. Shown are means±s.e.mean of three preparations each. In both groups of oocytes, the values were sequentially measured (1) in Ringer solution; (2) 5 min after the addition of L-arginine; (3) after repeated washout in Ringer solution and 10 min after the addition of AMT; and (4) 5 min after the addition of L-arginine in the presence of AMT. AMT did not alter the magnitude of L-arginine-induced currents in hCAT-2B or hCAT1 expressing oocytes.

L-arginine transport correlated with an enhanced expression of mRNA for CAT-2B (Messeri Dreißig *et al*., 2000; Hammermann *et al*., 2000). Evidence that CAT-2B may mediate the LPS-activated L-arginine transport was also obtained in studies on a murine MΦ cell line (Kakuda *et al*., 1999). In the present experiments, the LPS-induced up-regulation of the CAT-2B mRNA was not affected by inhibition of NO synthesis during the culture period excluding that endogenous NO is critically involved in the up-regulation of the expression of the CAT-2 gene. Despite the enhanced expression of CAT-2B, the LPS-stimulated L-arginine transport was abrogated in cells treated with NOS inhibitors indicating that the NOS inhibitors may affect L-arginine transport rather than the expression of the transporters. This is also supported by the observation that short time exposure to AMT already caused an inhibition of L-arginine uptake. Thus, a 30 min preincubation with AMT was sufficient to produce almost the same concentration-dependent inhibition which had been seen after 20 h culture with AMT, and a significant inhibition of L-arginine uptake was even observed when AMT was only present during the acute uptake period.

For the inhibition of LPS-stimulated L-arginine transport by NOS inhibitors two possible mechanisms have to be discussed. (1) The NOS inhibitors may selectively inhibit the LPS-stimulated L-arginine transport *via* direct inhibitory effects at the LPS-activated transporter. (2) Inhibition of NO synthesis may be responsible for the reduced L-arginine uptake. If so, either NO could act as an activator of L-arginine transport or simply the L-arginine flow through the iNOS pathway may be responsible for the enhanced L-arginine uptake.

The present observations support the last assumption which is shown schematically in Figure 9. First, the NOS inhibitors appear to act at an intracellular site. This is indicated by the observations that a marked inhibitory effect was observed, when the cells had been pretreated (i.e. loaded) with the drugs, but L-arginine uptake was studied in the absence of the NOS inhibitors. The inhibition of L-arginine uptake was even more pronounced when the cells were preincubated for 30 min with AMT and the drug was not present during the uptake period than in the experiments in which AMT was present only in the uptake medium. Finally,

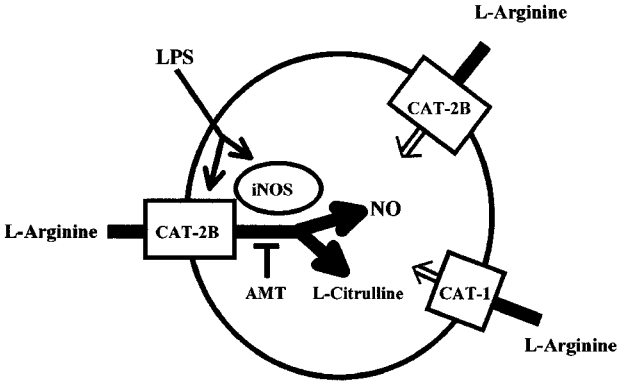

Figure 9 Scheme describing the possible link between LPS-stimulated L-arginine transport and iNOS induction in rat AMΦ. AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine, a specific iNOS inhibitor; CAT, cationic amino acid transporter.

when AMT was present only in the uptake medium its inhibitory potency depended on the time of incubation. Shortening of the incubation time (from usually 2 to 1 min) resulted in a marked attenuation of the effect of AMT (Figure 4). These observations in particular argue against a direct inhibitory effect of AMT at the extracellular site of the transporter. The observation that the inhibitory effect of AMT on L-arginine uptake was independent of the [^{3}H]-L-arginine concentration in the uptake medium argues also against a direct competitive interaction between AMT and L-arginine at the extracellular side of the transporter. The fact that AMT showed the same potency for the inhibition of the LPS-stimulated nitrite accumulation and the LPS-stimulated L-arginine uptake suggests that inhibition of NO synthesis might be responsible for the reduction of L-arginine uptake.

Furthermore, the observation that only the LPS-stimulated component of the L-arginine uptake was sensitive to NOS inhibitors, whereas the L-arginine uptake in control AMΦ showing low NO synthesis was only marginally affected, supports also the conclusion that L-arginine uptake in rat AMΦ is linked to the actual NO synthesis, in particular, as the transporter CAT-2B which is up-regulated by LPS appears to be of significance already in AMΦ cultured under control conditions. However, a stimulatory effect of NO on the L-arginine transport can be excluded as the NO releasing compound DETA-NONOate did not reverse the inhibitory effect of the NOS inhibitor. Therefore, it is concluded that the L-arginine uptake in rat AMΦ is stimulated by the substrate flow through the iNOS pathway (Figure 9).

Finally, the experiments, in which L-arginine transport in *Xenopus laevis* oocytes expressing either hCAT-1 or hCAT-2B was studied by electrophysiological techniques, demonstrated clearly that AMT does not directly affect L-arginine transport mediated by these CATs which are the human homologues of the CATs expressed in rat AMΦ (Racké *et al*., 1998; Messeri Dreißig *et al*., 2000; Hammermann *et al*., 2000). In *Xenopus laevis* oocytes expressing either hCAT-1 or hCAT-2B, addition of extracellular L-arginine induced a membrane depolarization which was not seen in control cells. This L-arginine-induced and CAT-mediated membrane depolarization was not affected by AMT. When E_M in oocytes expressing hCAT-1 or hCAT-2B was held constant, an L-arginine-induced current could be measured which was also not affected by AMT. It should be mentioned that in order to get clear signals in these electrophysiological experiments the concentration of L-arginine in the extracellular medium is relatively high (1 mM). Therefore, some uptake studies in rat AMΦ had also been carried out using 1 mM [^{3}H]-L-arginine. In rat AMΦ pretreated with 100 μM AMT the LPS-induced up-regulation of the transport of 1 mM [^{3}H]-L-arginine was abolished, but AMT in the same concentration did not affect the CAT-mediated transport of 1 mM L-arginine in oocytes. Therefore, it can be concluded that AMT, at concentrations inhibiting LPS-stimulated L-arginine uptake in rat AMΦ, does not directly affect CAT-mediated L-arginine transport.

In conclusion, since AMT appears not to interfere with L-arginine transport at the transporter site, its inhibitory effect on iNOS may be responsible for the inhibition of the LPS-activated L-arginine uptake in rat AMΦ. Since NO, however, does not stimulate L-arginine transport, it is concluded that the actual NO synthesis, i.e. the flow of L-arginine through the NOS pathway, may determine L-arginine uptake. The

present observations therefore suggest a very close link between LPS-induced iNOS and the LPS-activated L-arginine transporter, most likely CAT-2B (Figure 9). A functionally close link between CAT-2B-mediated L-arginine transport and iNOS-mediated NO synthesis has recently also been demonstrated in MΦ of mice as in MΦ from CAT-2 knockout mice induction of iNOS did not result in an increased NO synthesis (MacLeod *et al*., 1999).

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