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Expression of inducible nitric oxide synthase in mice: Pharmacological evaluation of adenosine receptor agonists

Shabbir M. Moochhala [a], Wei-Min Hon [b], Vikram J.S. Chhatwal [a], Hoon-Eng Khoo [b,*]

[a] *Department of Surgery, National University of Singapore, Singapore 119260, Singapore*
[b] *Department of Biochemistry, National University of Singapore, Singapore 119260, Singapore*

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Abstract

Inhibition of inducible nitric oxide (NO) synthase during endotoxaemia may be of therapeutic value. We have previously shown that pretreatment of mice with adenosine receptor agonists 1 h before lipopolysaccharide administration results in a dose-dependent reduction of plasma nitrite and nitrate (NO_x^-) levels. This report examines the effects of adenosine receptor agonists, 5′-*N*-ethylcarboxamidoadenosine (NECA), N^6-cyclohexyladenosine (CHA), *R*-phenylisopropyl-adenosine (*R*-PIA) and 5′-(*N*-cyclopropyl)carboxamidoadenosine (CPCA), on the level of inducible NO synthase expression in a model of liver inflammation induced by lipopolysaccharide. Following lipopolysaccharide administration (10 mg/kg, i.p.), liver mRNA expression peaked at 3 h and declined to 35% of maximal level after 24 h. Pretreatment with adenosine receptor agonists (0.001 mg/kg to 5 mg/kg, i.p.) depressed inducible NO synthase mRNA expression significantly. Down-regulation of inducible NO synthase mRNA expression corresponded with changes in plasma NO_x^- level as well as activity of NO synthase in the liver. Administration of *R*-PIA (5 mg/kg, i.p.) increased the survival of animals injected with a lethal dose of lipopolysaccharide. Thus adenosine receptor agonists may useful as anti-inflammatory agents in the treatment of endotoxaemia.

Keywords: Nitric oxide (NO) synthase; Nitric oxide (NO); mRNA expression; Adenosine; Anti-inflammatory

1. Introduction

Nitric oxide (NO) is a short-lived free radical, synthesized from the terminal guanidino nitrogen atom(s) of L-arginine by NO synthase (EC 1.14.13.39) (Moncada et al., 1991). There are at least 3 different NO synthase isoforms: The neuronal NO synthase, endothelial NO synthase and inducible NO synthase (Knowles and Moncada, 1994). The neuronal and endothelial types are constitutively expressed in neuroectodermal and endothelial cells respectively, and their activities are Ca^{2+}/calmodulin dependent (Busse and Mülsch, 1990a). Constitutive NO synthase generates low concentrations of NO which plays an important role in the physiological regulation of blood flow, blood pressure, platelet aggregation and neurotransmission (Schmidt and Walter, 1994). Inducible NO synthase is expressed only after cell activation by immunological stimuli such as bacterial endotoxin and cytokines in macrophages and related cell types (Stuehr et al., 1989), hepatocytes (Wood et al., 1993), fibroblasts (Willis et al., 1994), smooth muscle cells (Busse and Mülsch, 1990b), astrocytes (Endoh et al., 1994) and epithelial cells (Goureau et al., 1994). Inducible NO synthase does not typically require added Ca^{2+}/calmodulin for activity. Induction of inducible NO synthase will cause prolonged NO synthesis which is cytostatic or cytotoxic for invading microorganisms and tumor cells (Langrehr et al., 1993).

Considerable interest in the over-production of NO by inducible NO synthase in patients with septic shock has arisen, because this gaseous metabolite seems to contribute to the severe hypotension as well as direct tissue injury seen in these patients (Vallance and Moncada, 1993). Lipopolysaccharide is considered the most important exogenous mediator of septic shock. Binding of lipopolysaccharide to phagocytic cells, endothelial cells and platelets results in the release of various categories of endogenous mediators of septic shock (Welbourn and Young, 1992). Intravenous administration of lipopolysaccharide in animals and man produces a shock-like syndrome (Suffredini et al., 1989) and has been shown to induce inducible NO synthase expression (Wang et al., 1995). Excess production of NO in response to lipopoly-

* Corresponding author. Tel.: (65) 772-3250; Fax: (65) 779-1453.

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saccharide has also been implicated in the pathogenesis of other immunologically-mediated diseases such as rheumatoid arthritis (Farrel et al., 1992).

Since endogenous adenosine, released by damaged cells and tissues, or pharmacologically active adenosine analogues have been shown to have anti-inflammatory or immunosuppressive effects (Cronstein, 1994), we investigated the effects of these agents on inducible NO synthase expression. The actions of adenosine are mediated mainly by at least two distinct adenosine receptor types, designated as adenosine A_1 and A_2 receptors (Londos et al., 1980). Adenosine was found to inhibit a variety of neutrophil functions, including superoxide production and adhesion to mesenteric and myocardial vascular endothelium after ischemic-reperfusion injury (Grisham et al., 1989). Adenosine also inhibited phagocytosis of immunoglobulin-coated particles (Salmon et al., 1993) and secretion of the complement component C2 (Lappin and Whaley, 1984) by stimulated monocytes. Recently, adenosine and its analogues were found to inhibit production of tumour necrosis factor (TNF) by human monocytes (Le Vraux et al., 1993). Furthermore, we have shown that intraperitoneal injection of adenosine receptor agonists reduced lipopolysaccharide-induced plasma nitrite and nitrate (NO_x^-, the inactive end products of the NO pathway) concentrations in mice (Hon et al., 1995). In addition, a previous study has shown that inducible NO synthase expression was markedly increased in the liver of lipopolysaccharide-treated rats (Liu et al., 1993). To provide insights into the mechanism of action of adenosine receptor agonists on inducible NO synthase induction, the effects of the adenosine receptor agonists on the synthesis of liver inducible NO synthase mRNA and liver inducible NO synthase activity in lipopolysaccharide-treated mice were studied. Expression of inducible NO synthase mRNA in the liver was quantitated by competitive reverse transcription polymerase chain reaction (RT-PCR) (Siebert and Larick, 1992). Liver inducible NO synthase activity was also detected using the NADPH-diaphorase staining method which has been extensively used on fresh frozen specimens of a variety of tissues to label NO synthase (Chhatwal et al., 1994).

2. Materials and methods

2.1. Materials

Taq DNA polymerase and Trizol reagent were purchased from Gibco BRL (Grand Island, NY, USA). StrataScript reverse Transcriptase was from Stratagene (La Jolla, CA, USA). Deoxynucleotides (dNTPs) and recombinant ribonuclease inhibitor (rRNAsin) were from Promega (Madison, WI, USA). Mouse inducible (macrophage) NO synthase 3′ and 5′ gene-specific primers and mouse inducible NO synthase PCR MIMIC (competitive DNA fragment for quantitative RT-PCR) were from Clontech (Palo Alto, CA, USA). *R*-phenylisopropyladenosine (*R*-PIA) and 5′-(*N*-cyclopropyl)-carboxamidoadenosine (CPCA) were obtained from Research Biochemicals (Natick, MA, USA). Geneclean II kit was obtained from BIO 101 (La Jolla, CA, USA). Chloroform, isopropanol, zinc powder and cadmium acetate were from Merck (Darmstadt, Germany). LPS from *E. coli* (0127:B8 and 055:B5), sodium nitrite, dimethylsulfoxide (DMSO), diethylpropanolcyanate, sulfanilic acid, *N*-(1-naphthyl)ethylenediamine dihydrochloride, 5′-*N*-ethylcarboxamidoadenosine (NECA), and N^6-cyclohexyl-adenosine (CHA) and other chemicals were purchased from Sigma (St. Louis, MO, USA). Lipopolysaccharide was prepared in pyrogen-free 0.9% NaCl. NECA, *R*-PIA, CPCA and CHA were prepared as stock solutions in 4% DMSO in saline and diluted to the required concentration with saline.

2.2. Treatment of animals

Male Swiss mice weighing 20–28 g which had been starved overnight (18–24 h) but allowed free access to water received a single injection of lipopolysaccharide (5 mg/kg, i.p.). Control animals received an equivalent volume of 0.9% NaCl. At the indicated times after lipopolysaccharide administration, animals were anaesthetised with ether and bled from their axillary vessels. The blood was collected in heparinized tubes and centrifuged at $3000 \times g$ for 10 min. The plasma was transferred into clean tubes and stored at −70°C. The livers of the animals were removed and stored immediately at −70°C until use. To study the effect of the adenosine receptor agonists on inducible NO synthase expression, different groups of animals were injected i.p. with different adenosine receptor agonists one hour before administration of lipopolysaccharide. Mice were then sacrificed 8 h after endotoxin administration; the time corresponding to the NO_x^- (nitrite and nitrate) peak.

2.3. Extraction of total RNA

100 mg of liver tissue was homogenised in 1 ml of Trizol reagent using a glass–Teflon homogeniser. Total RNA was extracted according to the manufacturer's protocol. Briefly, the homogenate was mixed with chloroform, centrifuged in the cold and RNA was then precipitated from the aqueous phase by isopropanol. The RNA pellet was washed once in 70% ethanol, dried, and dissolved in diethylpropanolcyanate-treated water. The RNA was quantitated by spectrophotometry, and stored at −70°C. The yield was 80–100 μg of total RNA per 100 mg of liver tissue. Integrity of the total RNA was checked by electrophoresis in a 1% formaldehyde–agarose RNA gel.

2.4. Reverse transcription (RT)

5 μg of total RNA from each sample was reverse transcribed to cDNA by incubating with 50 mM Tris–HCl,

pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 30 units rRNAsin, 50 pmol oligo-dT primers, 250 μM each of dNTPs and 50 units of Stratascript reverse transcriptase in a final volume of 50 μl for 1 h at 37°C. The reaction was stopped by heating the mixture at 95°C for 10 min. The RT-mixture was then stored at −20°C until use.

2.5. Polymerase chain reaction (PCR)

PCR was performed by incubating 3 μl of RT-mixture with 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, 1.5 units *Taq* polymerase, 50 μM of each dNTP, 0.4 μM 3′ and 5′ murine inducible NO synthase gene-specific primer in a final volume of 25 μl for 30 cycles (1 min at 95°C, 1 min at 65°C, 1.5 min at 72°C) using a Hybaid Omni gene system. Before the first cycle, there was an initial denaturation step of 10 min at 95°C and an additional elongation step of 10 min at 72°C followed the 30 cycles.

Oligonucleotide primers for inducible NO synthase were 5′CCCTTCCGAAGTTTCTGGCAGCAGC3′ (sense) and 5′GGCTGTCAGAGCCTCGTGGCTTTGG3′ (antisense) which corresponded to the murine macrophage inducible NO synthase sequence.

2.6. Quantitation of mRNA using competitive RT-PCR

For mRNA quantitation, a constant amount of the sample cDNA was coamplified with a known concentration of the competitor DNA in the PCR cycles. Competitive DNA, the MIMIC, obtained from the supplier (Clontech) was a non-homologous DNA fragment derived from the V-*erb* B gene to which the pair of murine inducible NO synthase primer template had been added. To determine the appropriate amount of inducible NO synthase MIMIC to be used in the PCR amplification, a preliminary experiment was done. A constant amount of cDNA transcribed from 5 μg of total RNA was coamplified in the presence of 10-fold serial dilutions of the MIMIC. The results of this experiment provided a fine-tuned, 2-fold dilution series of the MIMIC. The same amount of cDNA was then coamplified with the 2-fold MIMIC dilution series. The amount of MIMIC (0.005 amol) which provided an optical density ratio of 1:1 with the target was chosen for further use. After PCR, the products derived from the MIMIC and target were resolved on agarose gel, and the ratio of the optical density of the PCR product pairs, 497 bp inducible NO synthase and 400 bp MIMIC, were determined.

2.7. Analysis of PCR products

An aliquot (20 μl) from each PCR reaction was electrophoresed in a 2% agarose gel containing 0.4 mg/ml ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the relative intensity of the PCR bands on the gel photographs were analysed using the Bioprofil system of a photoimager (Vilber Lourmat, France).

2.8. DNA sequencing

The 497 bp inducible NO synthase band from the agarose gel was cut out and the DNA was isolated using the Geneclean II kit. The purified DNA was then sequenced using an Applied Biosystems 373A DNA sequencer (Foster City, CA, USA).

2.9. Measurement of plasma NO_x^- (NO_2^- and NO_3^-)

The concentration of NO_2^- and NO_3^-, the stable end products of NO oxidation, in plasma were determined based on the Greiss reaction, in which NO_2^- reacted with

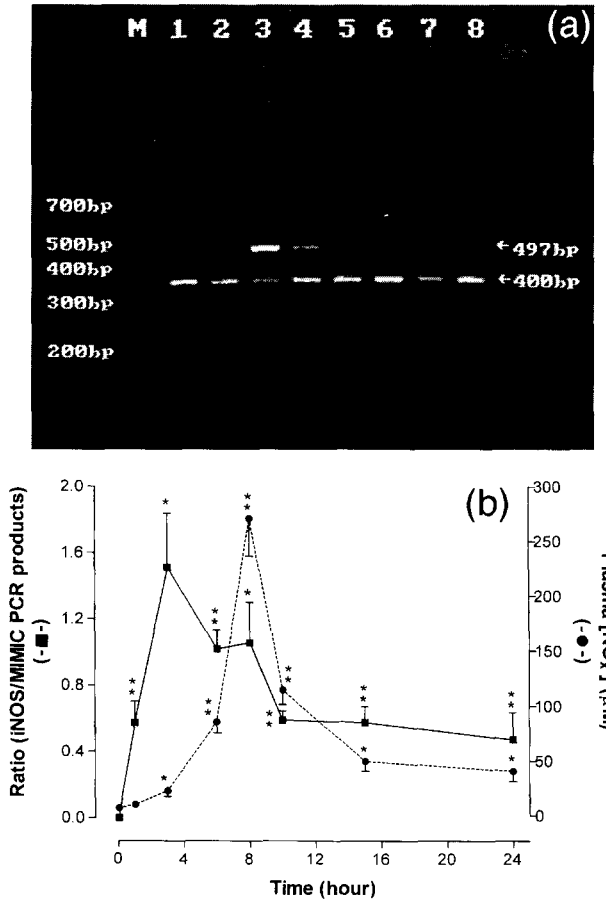

Fig. 1. (a) Quantitative analysis of time course induction of inducible NO synthase (iNOS) mRNA levels in lipopolysaccharide-stimulated mice. Ethidium bromide staining of RT-PCR products separated in 2% agarose gel. Lanes: M, molecular mass marker; 1, RT-PCR product from control mice; 2–8, RT-PCR products from mice treated with lipopolysaccharide for 1, 3, 6, 8, 10, 15 and 24 h. The 497 bp products from inducible NO synthase mRNA and the 400 bp products from MIMIC are indicated. (b) Effect of lipopolysaccharide on NO_x^- production and inducible NO synthase mRNA expression in mice as a function of time. Results are expressed as Mean ± S.E.M. ($n = 6$ for $[NO_x^-]$ and $n = 3$ for mRNA level). * $P < 0.01$ and ** $P < 0.001$ versus control.

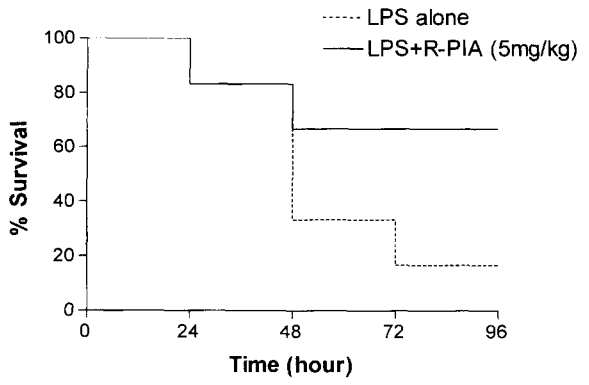

Fig. 2. Effect of *R*-PIA (5 mg/kg, i.p.) on survival of lipopolysaccharide-treated mice. 4 groups of six animals were used for mortality test. The animals that received only saline or *R*-PIA were used as control. * $P < 0.05$, *R*-PIA and lipopolysaccharide-treated versus lipopolysaccharide-treated.

1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4 forming a chromophore absorbing at 540 nm (Green et al., 1982). Copperised cadmium prepared according to the method of Rockett et al. (1992) was used to reduce all the NO_3^- in the plasma to NO_2^-. Plasma NO_x^- concentration was obtained by extrapolation from a sodium nitrite standard curve.

2.10. NADPH diaphorase histochemistry

Cryostat sections of liver were cut at 4 mm, mounted on slides and air dried. Tissue sections were fixed for 7 min in 4% paraformaldehyde/0.05 M phosphate buffer, pH 8.0. After rinsing with buffer, tissue sections were incubated in a mixture of β-NADPH (1 mg/ml) and nitroblue tetrazolium (0.1 mg/ml) in 0.1 M phosphate buffer (pH 8.0) containing 0.2% Triton X-100 for 45 min at 37°C. Staining was inhibited by incubating the sections with 1 M L-*N*-arginine-methylemine (L-NAME). The sections were rinsed with buffer before being mounted for observation. The stained tissue sections were viewed under a microscope and photographed under bright-field illumination.

2.11. Lethality test

To ascertain the potential role of adenosine receptor agonists in the prevention of death induced by lipopoly-

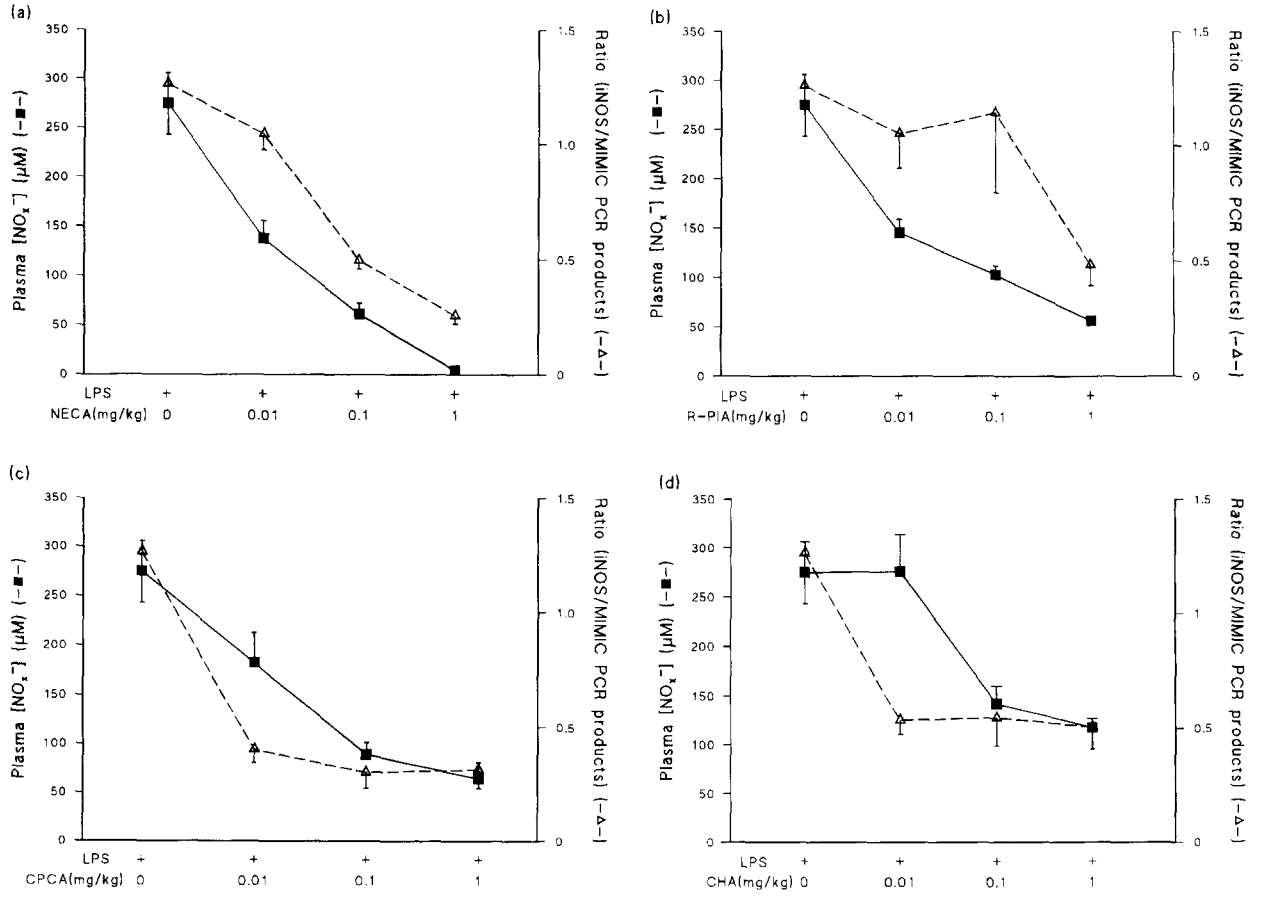

Fig. 3. Effects of adenosine receptor agonists on plasma NO_x^- and liver inducible NO synthase (iNOS) mRNA levels in lipopolysaccharide-treated mice. The adenosine receptor agonists used are (a) NECA, (b) *R*-PIA, (c) CPCA and (d) CHA. The agonists were injected into the animals 1 h before the administration of lipopolysaccharide and the animals were sacrificed 8 h later. Results are expressed as Mean ± S.E.M. ($n = 4$ for $[NO_x^-]$ and $n = 3$ for mRNA level).

saccharide *R*-PIA was selected since it was the most potent in reducing plasma NO_x^- level. A set of six male Swiss mice was treated with a single injection of lipopolysaccharide (from *E. coli* 055:B5, 100 mg/kg) intraperitoneally. Another set of animals was treated with *R*-PIA (5 mg/kg, i.p.) 1 h before the lipopolysaccharide administration. Animals which received saline or *R*-PIA alone were used as control. All the animals were observed over a period of 72 h for their survival.

2.12. Statistical analysis

Statistical significance of differences between treated and control groups was determined by an unpaired Student's *t*-test. Comparison between groups was by analysis of variance using the ANOVA analysis. The Wilcoxon's test was used for survival time comparison. A P value < 0.05 was taken to indicate statistical significance.

3. Results

After lipopolysaccharide (5 mg/kg) administration, all the animals looked sick but survived until the termination of the experiment. The PCR products of cDNA from lipopolysaccharide-injected mouse liver amplified using inducible NO synthase specific primers showed clear bands at the predicted size of 497 bp (Fig. 1a). This band was absent in the PCR-amplified products using total-RNA preparation as template. This indicates that the band originated from mRNA and not from genomic DNA. The 497 bp band on the agarose gel was then isolated, cleaned and sequenced. The sequence of the isolated band was 100% homologous to the mouse macrophage inducible NO synthase sequence.

Administration of lipopolysaccharide resulted in a time-dependent increase in the expression of liver inducible NO synthase mRNA (Fig. 1a and b). Expression of

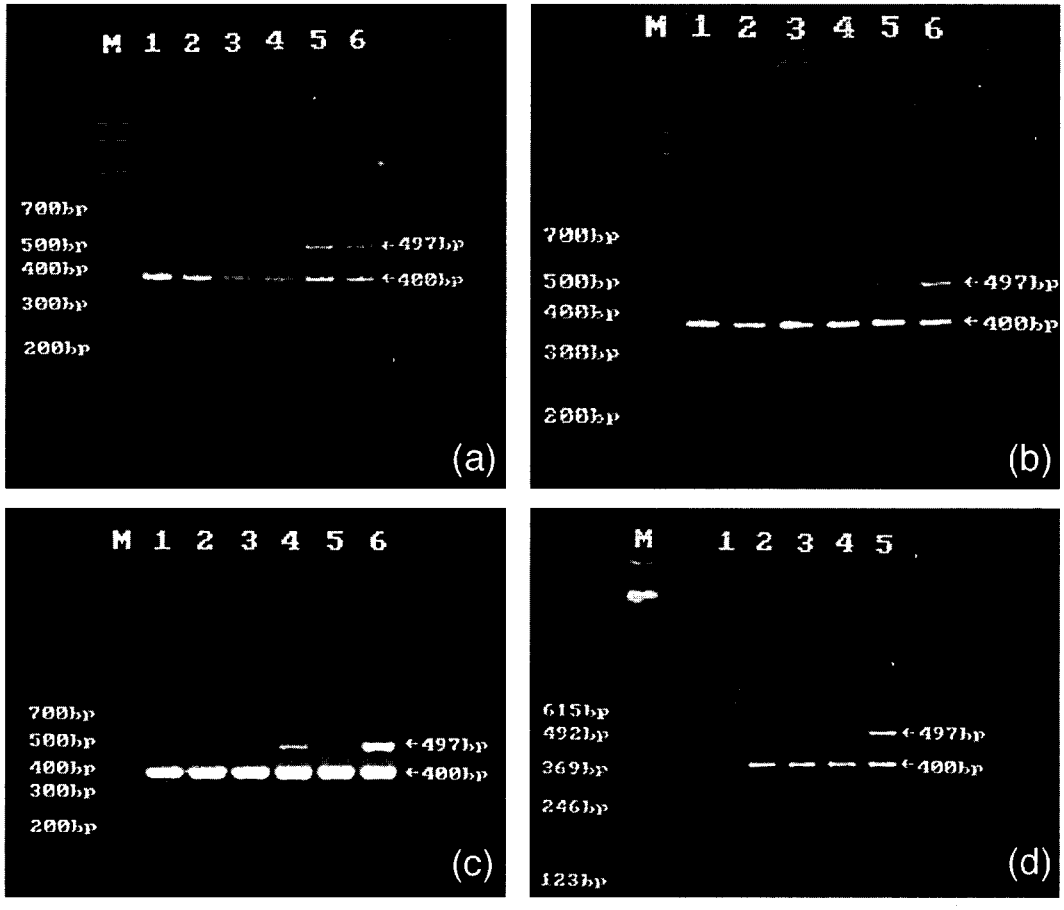

Fig. 4. Effects of adenosine agonists on inducible NO synthase mRNA level in lipopolysaccharide-treated animals. (a) Effect of NECA. Lanes: M, molecular mass marker; 1, RT-PCR product of control mice; 2–5, RT-PCR product of mice pre-treated with 1 mg/kg, 0.1 mg/kg, 0.01 mg/kg and 0.001 mg/kg of NECA before lipopolysaccharide administration; 6, RT-PCR product of mice treated with lipopolysaccharide alone. (b) Effect of *R*-PIA. Lanes: M, molecular mass marker; 1, RT-PCR product of control mice; 2–5, RT-PCR product of mice pre-treated with 5 mg/kg, 1 mg/kg, 0.1 mg/kg and 0.01 mg/kg of *R*-PIA before lipopolysaccharide administration; 6, RT-PCR product of mice treated with lipopolysaccharide alone. (c) Effect of CPCA. Lanes: M, molecular mass marker; 1, RT-PCR product of control mice; 2–5, RT-PCR product of mice pre-treated with 1 mg/kg, 0.1 mg/kg, 0.01 mg/kg and 0.001 mg/kg of CPCA before lipopolysaccharide administration; 6, RT-PCR product of mice treated with lipopolysaccharide alone. (d) Effect of CHA. Lanes: M, molecular mass marker; 1, RT-PCR product of control mice; 2–4, RT-PCR product of mice pre-treated with 2.5 mg/kg, 1 mg/kg and 0.5 mg/kg of CHA before lipopolysaccharide administration; 5, RT-PCR product of mice treated with lipopolysaccharide alone.

inducible NO synthase mRNA was quantitated by competitive RT-PCR as described in Materials and methods. Induction of liver inducible NO synthase mRNA by lipopolysaccharide was almost immediate. The level of liver inducible NO synthase mRNA reached a peak after 3 h and then slowly declined to 35% of the maximal level after 24 h (Fig. 1a and b). The inducible NO synthase signal was absent or negligible in samples extracted from the livers of control mice (animals that only received saline). Liver inducible NO synthase mRNA expression was studied in parallel with systemic inducible NO synthase activity as determined by the concentrations of the inactive end products of the NO pathway, nitrates and nitrites in plasma. Plasma NO_x^- concentration increased significantly 6 h after the injection of lipopolysaccharide, reaching a peak 8 h later (38-fold basal level) and then declined slowly towards control levels by 24 h (Fig. 1b). Control mice had a basal plasma NO_x^- concentration of 7.9 ± 1.2 μM ($n = 8$).

Our previous report (Hon et al., 1995) had shown that pre-treatment of mice with adenosine receptor agonists significantly reduced the production of plasma [NO_x^-] in lipopolysaccharide-treated mice. The agonists were chosen for their different affinities for adenosine A_1 and A_2 receptors. NECA has equal affinity for both adenosine A_1 and A_2 receptors, whereas *R*-PIA and CHA have higher affinity for adenosine A_1 receptor while CPCA is specific for the adenosine A_2 receptor (Bruns et al., 1986). Adenosine receptor agonists reduced the lipopolysaccharide-induced plasma NO_x^- production dose-dependently with the IC_{50} values varying from 85 to 100 ng/kg. The rank order of potency based on IC_{50} values were determined as follows: NECA $\geq$ *R*-PIA > CPCA > CHA. This rank order of potency was not characteristic of adenosine A_1 nor A_2 receptor. Our results also showed that injection of an adenosine receptor agonist before the treatment of mice with lipopolysaccharide, will reduce mortality. Fig. 2 shows that mortality rates of lipopolysaccharide-treated mice decreased if *R*-PIA (5 mg/kg, i.p.) was injected 1 h before the animals were subjected to a lethal dose of lipopolysaccharide. Animals injected with saline or *R*-PIA alone were used as control. *R*-PIA was chosen since it is one of the most potent in reducing plasma NO_x^- level.

Analysis using a competitive RT-PCR method revealed a remarkable suppression of liver inducible NO synthase mRNA expression in lipopolysaccharide-stimulated mouse upon pre-treatment of the animals with various adenosine receptor agonists. Fig. 3 shows the effects of the adenosine receptor agonists on the synthesis of inducible NO synthase mRNA in lipopolysaccharide-treated mice. The adenosine receptor agonists alone had no effect on the basal liver inducible NO synthase mRNA level (data not shown). Pre-treatment of animals with NECA and *R*-PIA resulted in a dose-dependent reduction of the lipopolysaccharide-induced inducible NO synthase mRNA expression (Fig. 3a and b and Fig. 4a and b). Injection of animals

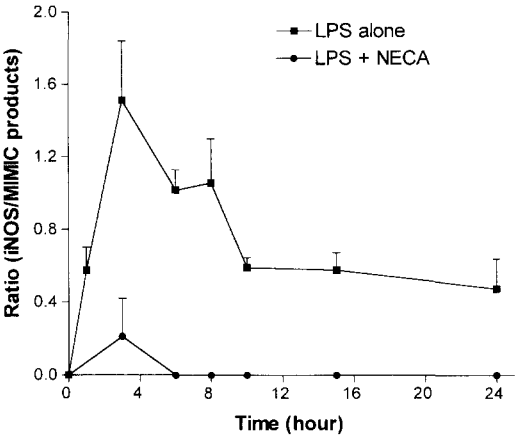

Fig. 5. Time-course of suppression of liver-inducible NO synthase (iNOS) mRNA level in lipopolysaccharide-treated mice by NECA (0.1 mg/kg). Inducible NO synthase mRNA level in lipopolysaccharide-treated (■) and lipopolysaccharide- and NECA-treated (•) mice. Results are expressed as mean ± S.E.M. ($n = 3$). Two way ANOVA indicates that the inducible NO synthase mRNA level varies significantly with time ($P < 0.0001$) and treatment with NECA ($P < 0.0001$).

with a low dose of NECA (0.001–0.01 mg/kg) did not have any significant effect on lipopolysaccharide-induced inducible NO synthase mRNA expression. At higher doses (0.1 mg/kg and 1 mg/kg) of NECA, the inducible NO synthase mRNA levels was reduced by 40% and 21% of the lipopolysaccharide-induced level (Fig. 3a and Fig. 4a). Administration of *R*-PIA (0.01–5 mg/kg, i.p.) resulted in the reduction of inducible NO synthase mRNA levels dose-dependently to 34–90% of the lipopolysaccharide-induced level (Fig. 3b and Fig. 4b). Administration of CPCA and CHA reduced the mRNA levels significantly at all concentrations used (Fig. 3c and d, and Fig. 4c and d). NECA at a concentration of 0.1 mg/kg was chosen for further investigation on the effects of NECA on the time course of induction of inducible NO synthase mRNA by lipopolysaccharide. The results showed that inducible NO synthase mRNA expression induced by lipopolysaccharideis down-regulated by NECA (0.1 mg/kg) for the whole time-course (Fig. 5).

NADPH diaphorase activity was detected in the mice liver sections as indicated by the blue staining in the cytoplasm of the cells (most of which are hepatocytes), while nuclear fast red was seen staining the nuclei. The staining intensity of the liver sections from lipopolysaccharide-stimulated animals was time-dependent. Liver sections from control animals were only very lightly stained (Fig. 6a). The liver sections from animals sacrificed 8 h after lipopolysaccharide treatment were intensively stained (Fig. 6b) while those from animals which had received adenosine receptor agonist (NECA, 1 mg/kg) treatment before lipopolysaccharide injection, showed very low NADPH diaphorase activity (Fig. 6c). The adenosine receptor agonists, *R*-PIA (1 mg/kg), CPCA (1 mg/kg) and NECA (1 mg/kg) also reduced the NADPH diaphorase staining in

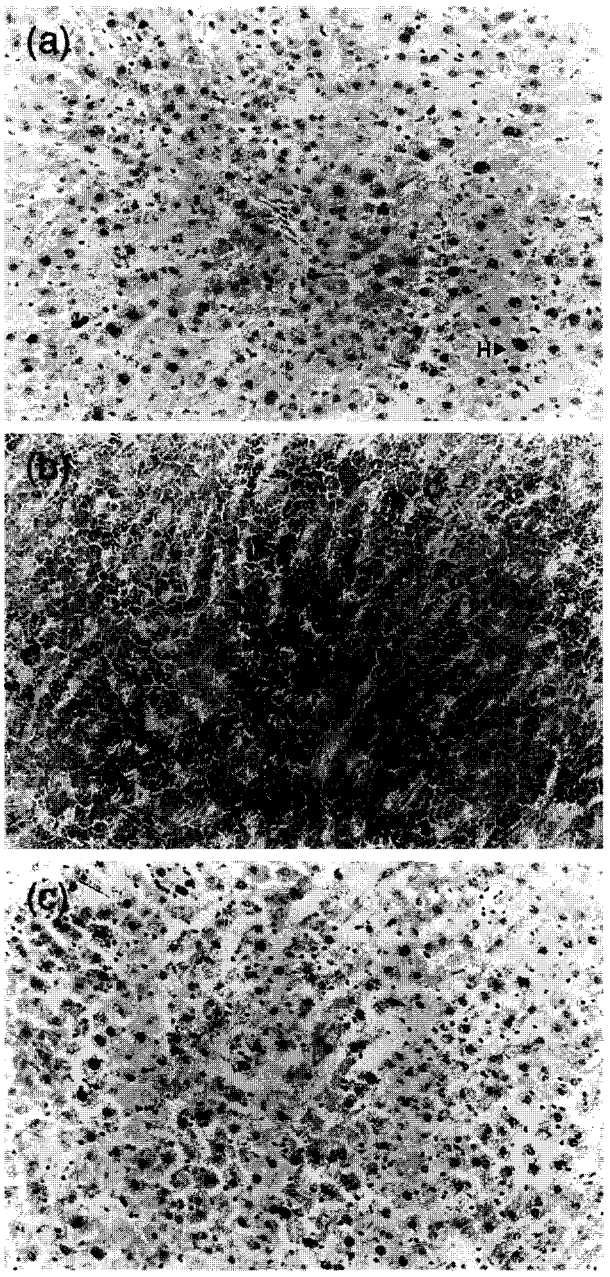

Fig. 6. NADPH-diaphorase staining of liver sections. (a) Liver section from control mice with low blue staining. H: Hepatocyte. (b) Liver section from mice treated with lipopolysaccharide (5 mg/kg) for 8 h, which was intensively stained with blue. (c) Liver section from mice pre-treated with 1 mg/kg NECA before the treatment with lipopolysaccharide for 8 h with low blue staining. The sections were photographed under bright field illumination at 200× original magnification.

the liver sections (data not shown). At these concentrations, the adenosine receptor agonists were able to inhibit the production of plasma $[NO_x^-]$ and the expression of liver inducible NO synthase mRNA significantly.

To prove that the NADPH diaphorase activity shown by the liver tissues is actually due to the activity of NO synthase, and not other enzymes, NO synthase specific inhibitor L-NAME (Rees et al., 1990) was incubated together with the staining mixture for the liver sections that had shown NADPH diaphorase activity. L-NAME (1 M) managed to eliminate most of the diaphorase activity thus confirming that the NADPH diaphorase activity shown in the liver sections was mainly due to the presence of inducible NO synthase and not other enzymes (data not shown). The low NADPH diaphorase activity shown in control liver was most probably due to the presence of a low amount of other enzymes, including the constitutive isoforms of NO synthase.

4. Discussion

These results demonstrate that intraperitoneal administration of lipopolysaccharide results in a marked enhancement of liver inducible NO synthase mRNA as analysed by competitive RT-PCR. Lipopolysaccharide has been previously shown to induce inducible NO synthase mRNA expression in liver (Liu et al., 1993) by the Northern blot technique. Competitive RT-PCR is an established method for the quantitative measurement of gene expression and it has been widely used (Gilliland et al., 1990). The time course induction of inducible NO synthase mRNA by lipopolysaccharide using a competitive RT-PCR method has not been previously reported. The inducible NO synthase mRNA was absent or negligible in the liver from control mice. Induction of liver inducible NO synthase mRNA by lipopolysaccharide was almost immediate (reaching the peak level 3 h after lipopolysaccharide treatment) and then slowly declined. This correlates well with the increase in liver inducible NO synthase activity as well as systemic NO production in the same animal model. Since the induction of inducible NO synthase activity requires de novo protein synthesis over several hours, this may explain why the plasma $[NO_x^-]$ and NADPH-diaphorase staining reached maximal level a few hours (peak at 8 h after lipopolysaccharide treatment) after mRNA induction.

Treatment with various concentrations of the adenosine receptor agonists caused a remarkable suppression of the lipopolysaccharide induced inducible NO synthase mRNA expression as shown by the decrease in the signal of the 497 bp inducible NO synthase band. However, the adenosine receptor agonists did not all act in similar manner. At low concentrations of NECA and *R*-PIA when there was no or very low inhibition of lipopolysaccharide-induced inducible NO synthase mRNA expressions, there was strong inhibition of plasma NO_x^- production. This implies that NECA and *R*-PIA may have post-translational inhibitory effects on inducible NO synthase enzyme activity other than inducible NO synthase mRNA induction. They

might directly or indirectly inactivate the inducible NO synthase enzyme by affecting its active site or by reducing the availability of the substrates and cofactors for the enzyme. On the other hand, CPCA and CHA might be more effective in reducing gene expression than inhibiting the existing enzyme activity and therefore, the plasma NO_x^- production remained high even when the mRNA level was low.

By using the NADPH-diaphorase staining method, adenosine receptor agonists were shown to inhibit inducible NO synthase activity in the lipopolysaccharide-treated mice liver sections. Since there has been some controversy over the use of the NADPH-diaphorase method to indicate the presence of inducible NO synthase activity (Persson et al., 1993), we confirmed that the NADPH diaphorase activity detected in the liver sections was due to the presence of NO synthase because it was inhibitable by L-NAME, a NO synthase specific inhibitor.

Our previous findings showed that treatment with adenosine receptor agonists prevented the rise in circulating NO_x^- levels in lipopolysaccharide-challenged mice and protected mice against a challenge with a lethal dose of endotoxin (Hon et al., 1995). The adenosine receptor agonists reduced the plasma [NO_x] in a dose-dependent manner with the potency rank as NECA ≥ *R*-PIA > CPCA > CHA. The observed response did not fall clearly into one of the two expected patterns for adenosine A_1 or A_2 receptors. The adenosine receptors, A_1 and A_2, display different affinities for adenosine and adenosine analogues. Ligand-binding to either receptor leads to changes in cellular 3′,5′-adenosine cyclic monophosphate (cAMP) concentrations (Olah and Stiles, 1992). Adenosine A_1 receptors have a high affinity for adenosine and binding of adenosine A_1 receptors inhibits cAMP generation induced by other ligands. Conversely, adenosine A_2 receptors have lower affinity for adenosine and ligand-binding stimulates cAMP generation which activates various cellular functions. Increased levels of cAMP have been shown to prevent polymorphonuclear leukocytes (PMNs) adherence to endothelial cells as well as decreased superoxide production and phagocytic activity, resulting in some possible anti-inflammatory effects (Mitsuyama et al., 1993). Results obtained from other laboratories indicated that the anti-inflammatory effects of adenosine and its analogues are mainly mediated through the adenosine A_2 receptors. For example, Cronstein et al. (1985) showed that adenosine inhibited neutrophil superoxide production via the adenosine A_2 receptor. In addition, Xiong et al. (1992) showed that adenosine and its receptor agonists induced metallothionein protein synthesis and mRNA expression in rat liver presumably via adenosine A_2 receptor using the cAMP pathway. Therefore, the effect of adenosine receptor agonists on the induction of inducible NO synthase might partly be an effect of increased cAMP through binding to the adenosine A_2 receptor. Even though there are reports showing that cAMP and cAMP-eliciting agonists enhanced inducible NO synthase expression in cells such as messengial cells (Kunz et al., 1994), Kupffer cells (Gaillard et al., 1992) and vascular smooth muscle cells (Koide et al., 1993), these effects were observed in vitro and therefore cannot be compared directly with our in vivo results. Besides, our studies show that the effects of adenosine receptor agonists on inducible NO synthase gene expression is neither adenosine A_1 nor A_2 receptor specific. Thus there is a possibility that adenosine receptors other than A_1 and A_2, such as the adenosine A_3 receptor (Zhou et al., 1992) might be involved in the process. The effects of A_3 specific agonists are presently under investigation. In addition, in the liver, inducible NO synthase may be induced in hepatocytes, Kupffer cells or other infiltrating inflammatory cells. Thus the results that we obtained which do not fit into the expected pattern for adenosine receptor subtypes might reflect the heterogeneity of receptors in different cell types.

Our results imply that adenosine receptor agonists may have direct inhibitory effects on lipopolysaccharide-induced inducible NO synthase gene expression. Adenosine receptor agonists may decrease the rate of transcription of the inducible NO synthase gene or they may also have some post-transcriptional modification effects on inducible NO synthase mRNA. They may be able to reduce the stability of the mRNA in the cells by an unknown mechanism and therefore reduce the half-life of the inducible NO synthase mRNA. However, since this is an in vivo study, we cannot confirm whether the inhibitory effects of adenosine receptor agonists were via a direct effect on inducible NO synthase induction or whether additional mechanisms could be involved. In the septic shock syndrome, many chemical mediators, such as cytokines, leukotrienes, and prostaglandins, are involved in the development of endotoxin shock (Glauser et al., 1991). Adenosine receptor agonists could interfere with the effects of other cytokines, like γ-interferon (γ-IFN), interleukin-1 (IL-1) and TNF, which are known to induce the Ca^{2+}-independent NO synthase (Hevel et al., 1991), such that the reduction of inducible NO synthase might be the consequence of reduced cytokine synthesis. Further work with a murine macrophage cell line has been started in our laboratory to study the role of adenosine and its receptor agonists on inducible NO synthase induction in vitro.

NO has been implicated in endotoxin shock as well as other immunologically-mediated diseases in several animal models and in man. Although it has been shown that low amounts of NO are beneficial in endotoxin shock, possibly by maintaining the vasodilator tone necessary for organ perfusion (Nava et al., 1991), the large amounts of NO induced by cytokines and lipopolysaccharide are detrimental and contribute to the observed mortality (Wright et al., 1992). Since NO generation by inducible NO synthase seems to be a major mechanism in host defence, adenosine agonists which can reduce inducible NO synthase induction may possibly become useful anti-inflammatory drugs.

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