Effects of aminoguanidin on nitric oxide production induced by inflammatory cytokines and endotoxin in cultured rat hepatocytes

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Abstract

AIM To study the effects of aminoguanidine (AG) and two L-arginine analogues Nω-nitro-L-arginine methyl ester (L-NAME) and Nω-nitro-L-arginine (L-NNA) on nitric oxide (NO) production induced by cytokines (TNF-α, IL-1β, and IFN-γ) and bacterial lipopolysaccharide (LPS) mixture (CM) in the cultured rat hepatocytes, and examine their mechanisms of action.

METHODS Rat hepatocytes were incubated with AG, L-NAME, L-NNA, Actinomycin D (ActD) and dexamethasone in a medium containing CM (LPS plus TNF-α, IL-1β, and IFN-γ) for 24 h. NO production in the cultured supernatant was measured with the Griess reaction. Intracellular cGMP level was detected with radioimmunoassay. RESULTS NO production was markedly blocked by AG and L-NAME in a dose-dependent manner under inflammatory stimuli condition triggered by CM in vitro. The rate of the maximum inhibitory effects of L-NAME (38.9%) was less potent than that obtained with AG (53.7%, P<0.05). There was no significant difference between the inhibitory effects of AG and two L-arginine analogues on intracellular cGMP accumulation in rat cultured hepatocytes. Non-specific NOS expression inhibitor dexamethasone (DEX) and iNOS mRNA transcriptional inhibitor ActD also significantly inhibited CM-induced NO production. AG (0.1 mmol·L⁻¹) and ActD (0.2 ng·L⁻¹) were equipotent in decreasing NO production induced by inflammatory stimuli in vitro, and both effects were more potent than that induced by non-selectivity NOS activity inhibitor L-NAME (0.1 mmol·L⁻¹) under similar stimuli conditions (P<0.01).

CONCLUSION AG is a potent selective inhibitor of inducible isoform of NOS, and the mechanism of action may be not only competitive inhibition in the substrate level, but also the gene expression level in rat hepatocytes.

INTRODUCTION

The pathogenesis of inflammatory sepsis and multiple autoimmune diseases (such as insulin-dependent diabetes mellitus, arthritis and viral hepatitis) is linked to the overproduction of nitric oxide (NO), a potentially toxic molecule, which is likely to be responsible in part for the cytotoxicity and mutagenicity of the inflammatory process. NO has been reported to cause DNA damage and mutations, and to induce apoptosis in several cell types for example macrophages, hepatocytes and pancreatic β-cells[1-7]. The analogues of L-arginine, Nω-nitro-L-arginine (L-NNA) or Nω-nitro-L-arginine methyl ester (L-NAME) may be useful in treating patients with the above diseases. However, the known inhibitors of the inducible enzyme can also exert their actions on the constitutive isoform NO synthase (cNOS), which resulted in an inappropriate vasodilatation or hypertension[8-10]. Thus, to inhibit selectively the inducible NOS (iNOS) may yield less side effects.

Aminoguanidine (AG) has effects on several enzyme systems, it interferes with non-enzymic glycosylation, leading to its potential use as a treatment for the complications of diabetes. Furthermore, recent work has showed that AG can also be used as a novel selective iNOS inhibitor in rat isolated vessels[11-13], although the mechanism
underlying this is unclear. The aim of this study was to determine the effects of AG and two nonselective NOS inhibitors L-NNA and L-NAME, on the NO production and cGMP release induced by cytokines and LPS in rat cultured hepatocytes.

MATERIALS AND METHODS

Reagents

Collagenase (Type IV, 340kU·g⁻¹), bovine insulin, lipopolysaccharide (LPS, E. coli 0111:B4), N⁶-nitro-L-arginine methyl ester (L-NAME), N⁶-nitro-L-arginine (L-NNA), aminoguanidine (AG), dexamethasone were purchased from Sigma Chemical Co.; human recombinant (rh) tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interferon-gamma (IFN-γ) were from Academy of Medical Sciences (Beijing), and Dulbecco’s modified Eagle’s medium (DMEM) from Gibco BRL; Bacille Calmétte-Guérin vaccine (BCG) was obtained from the National Vaccine and Serum Institute (Beijing), [³H]-radioimmunoassay kit for cyclic guanylate monophosphate (cGMP) was bought from Beijing Institute of Nuclear Research. All the reagents were diluted in medium and prepared freshly before use.

Isolation and culture of hepatocytes

Hepatocytes were harvested from male adult Wistar rats (weighing 180 g-220 g) using an in situ collagenase perfusion technique[14]. After inhalation anesthesia, the abdomens of the animals were opened and shaved, the portal vein was exposed and cannulated. Then the liver was perfused at 37 °C in situ first with a calcium-free phosphate-buffered saline solution (PBS). This perfusion was continued for 5 min, then it was switched to 0.5 g·L⁻¹ collagenase and 10 g·L⁻¹ bovine albumin in PBS buffer for 15 min. The liver was removed and the cells were combed gently in tissue culture medium. Hepatocytes were pelleted, washed, and separated from nonparenchymal cells by differential centrifugations at 50 × g. Viability of cells exceeded 90% as determined by trypan blue exclusion. Hepatocytes were plated onto 6-well plastic tissue-culture plates (1 × 10⁹ cells·L⁻¹ in each well). Medium in the control consisted of DMEM with L-arginine (0.5 mmol·L⁻¹), insulin (1 µmol·L⁻¹), Hepes (15 mmol·L⁻¹), L-glutamine, penicillin, streptomycin, and 100 mL·L⁻¹ low-endotoxin newborn calf serum. After overnight incubation, the medium was changed with a cytokines mixture (CM) containing LPS (10 µg·L⁻¹), IL-1β (100KU·L⁻¹), TNF-α (500KU·L⁻¹) and IFN-γ (100KU·L⁻¹). Other experimental conditions included addition of NO inhibitors (L-NAME, L-NNA or AG), actinomycin D (ActD) or dexamethasone (DEX) to the CM.

After primary cultures were maintained for 24 h at 37°C in 50 mL·L⁻¹ CO₂, hepatocytes or cultured supernatants were collected for nitrite and cGMP assays[15].

Determination of NO production and cGMP levels

To determine the amount of NO produced by hepatocytes, the culture supernatants were assayed for the stable oxidative product, nitrite, by an automated procedure based on the Griess reaction, as previously described[17]. The intracellular levels of cGMP were determined using a [³H]-labeled radioimmunoassay (RIA) kit, as described[14,15].

Statistical analysis

Data were expressed as x ± s. The significance of differences was determined with the Student’s t test. Statistical significance was established at a P value <0.05.

RESULTS

Effect of aminoguanidine and N⁶-nitro-L-arginine methyl ester on nitrite production

Being consistent with our previous results, the inflammatory factors induced a large amount of NO in a time- and dose-dependent manner in the primary culture of rat hepatocytes. Both L-NAME and AG inhibited LPS and cytokines-induced NO production (Table 1) in a dose-dependent fashion. On the other hand, AG completely blocked NO production stimulated by inflammatory factors in rat hepatocytes, however, the rate of the maximum inhibitory effects of L-NAME (38.9%) was less potent than that obtained with AG (53.7%, P<0.05).

Table 1 Inhibitory effect of aminoguanidine and L-NAME on CM stimulated in vitro nitrite production in primary cultured rat hepatocytes

| Dose (µmol·L⁻¹) | n | c (NO⁻) (µmol·L⁻¹) | Inhibition rate (%) | Dose (µmol·L⁻¹) | n | c (NO⁻) (µmol·L⁻¹) | Inhibition rate (%) |
|-----------------|---|-------------------|-------------------|-----------------|---|-------------------|-------------------|
| Control         | 6 | 6.2±1.0           |                   | Control         | 6 | 6.5±1.3           |                   |
| CM              | 16| 12.3±4.1          |                   | CM              | 8 | 12.6±3.7          |                   |
| CM+Aminoguanidine | 16| 5.5±2.1           |                   | CM+Aminoguanidine | 8 | 5.8±2.8           |                   |
| 0.1             | 1 | 9.6±3.9           | 22.0              | 0.1             | 9 | 10.1±1.9          | 19.8              |
| 1               | 16| 8.4±2.6           | 31.7              | 1               | 9 | 9.4±2.5           | 25.4              |
| 10              | 16| 6.9±2.8           | 44.0              | 10              | 9 | 8.5±3.0           | 32.5              |
| 100             | 16| 5.7±1.9           | 53.7              | 100             | 9 | 7.7±2.4           | 38.9              |
| 1000            | 16| 5.5±2.1           | 55.3              | 1000            | 9 | 7.8±2.8           | 38.1              |

CM (Cytokines mixture): IL-1β 10KU·L⁻¹, TNFα 500KU·L⁻¹, and IFNγ 100KU·L⁻¹ plus LPS 10 mg·L⁻¹; Cultured hepatocytes were stimulated in vitro with CM in the absence or presence of inhibitors (aminoguanidine or L-NAME) at various concentrations for 24 h. Amounts of nitrite in the supernatant were assayed 24 h after start of stimulation in vitro. *P ≤ 0.05, t = 6-16 rats (3 well for each treatment in each experiment); **P < 0.01, compared with control, ***P<0.05, ****P<0.01 compared with corresponding CM.
Effect of aminoguanidine, L-NNA and L-NAME on intracellular cGMP accumulation

To confirm that AG and two L-arginine analogues NOS inhibitor L-NAME and L-NNA, inhibit NO production induced by inflammatory stimuli, the effects of the three compounds on CM-induced accumulation of intracellular cGMP (a sensitive but non-specific index of NO production) were investigated. Two L-arginine analogues L-NAME and L-NNA almost completely inhibited cytokines-stimulated intracellular cGMP accumulation from 3.0±1.5 (pmol·L⁻¹) to 1.4±0.4 (pmol·L⁻¹) with L-NAME, and to 1.3±1.1 (pmol·L⁻¹) with L-NNA (Table 2). AG on intracellular cGMP accumulation was also parallel with NO production, although the inhibitory effect of AG seemed weaker as compared with that of the two L-arginine analogues to 1.48±0.8 (pmol·L⁻¹).

| Groups                        | n  | c (cGMP) (pmol·L⁻¹) | Inhibition rate (%) |
|-------------------------------|----|--------------------|--------------------|
| Control                       | 7  | 1.29±0.6           |                    |
| Cytokines mixture (CM)        | 12 | 3.01±1.5           | 62.5%              |
| CM+Aminoguanidine (0.1 mmol·L⁻¹) | 7  | 1.48±0.8           | 50.9               |
| CM+LNNNA (0.1 mmol·L⁻¹)       | 7  | 1.33±1.1           | 55.8               |
| CM+LNAME (0.1 mmol·L⁻¹)       | 7  | 1.36±0.4           | 54.8               |

CM (Cytokines mixture): IL-1β 10KU·L⁻¹, TNFα 500KU·L⁻¹, and IFNγ 100KU·L⁻¹ plus LPS 10 mg·L⁻¹; cultured hepatocytes were incubated in vitro with CM in the absence or presence of inhibitors (aminoguanidine, L-NAME, or L-NNA, respectively) for 24 h; Levels of intracellular cGMP were assayed 24 h after start of stimulation in vitro (CM or CM were co-incubated with inhibitors, respectively); x ± s, n = 7 rat or 12 rat (4 replicates for each treatment in each experiment); *P<0.05 compared with control, †P<0.01 compared with CM.

Comparison of inhibitory effects of aminoguanidine, L-NAME, DEX or ActD on nitrite production stimulated by CM in vitro

NO production was blocked by AG and L-NAME under inflammatory condition triggered by CM in vitro, AG (inhibition 52.2%) is a more potent inhibitor than L-NAME (35.5%, P<0.01, Table 3), the former completely blocked NO formation induced by CM in vitro. Similarly, both dexamethasone (DEX, 33.9%) and actinomycin D (ActD, 53.8%), inhibitors of non-specific NOS expression and iNOS mRNA transcription, also inhibited CM-induced NO production in rat hepatocytes. Furthermore, AG and ActD were equipotent in decreasing NO production triggered by inflammatory stimuli in vitro, and both effects more potent than that induced by L-NAME under similar stimuli condition (P<0.01).

| Groups                    | n  | c (NO) (µmol·L⁻¹) | Inhibition rate (%) |
|---------------------------|----|------------------|--------------------|
| Control                   | 12 | 6.0±2.4          |                    |
| Cytokines mixture (CM)    | 12 | 12.1±4.5         | 50.9               |
| CM+Dexamethasone (10 µmol·L⁻¹) | 12 | 8.1±2.3          | 33.9               |
| CM+L-NAME (0.1 mmol·L⁻¹)  | 12 | 7.8±1.6          | 35.5               |
| CM+Aminoguanidine (0.1 mmol·L⁻¹) | 12 | 5.8±1.1          | 52.2               |
| CM+Actinomycin D (0.2 ng·L⁻¹) | 15 | 5.7±2.3          | 53.8               |

CM (Cytokines mixture): IL-1β 10KU·L⁻¹, TNFα 500KU·L⁻¹, and IFNγ 100KU·L⁻¹ plus LPS 10 mg·L⁻¹; cultured hepatocytes were harvested from rats that control group stimulated in vitro with CM in the absence or presence of inhibitors (AG, L-NAME, ActD or DEX, respectively) for 24 h; the amount of nitrite in the supernatant was assayed 24 h after start of stimulation in vitro. x ± s, n = 12 or 15 rats (3 wells for each treatment in each experiment); †P<0.01, compared with control. *P<0.05, †P<0.01, compared with CM.

DISCUSSION

Recent work showed that AG, a nucleophile hydrazine compound, is structurally similar to L-arginine in that these compounds contain two chemically equivalent guanidino nitrogen groups and to L-arginine analogues that competitively inhibit NO synthase. The present study showed that AG completely prevents inflammatory stimuli induced formation of NO and confirmed that it is a potent inhibitor of the cytokine-inducible isoform NOS. On the other hand, administration of L-NAME failed to completely suppress the NO production induced by inflammatory stimuli in rat hepatocytes. This result was not associated with the previous observation[16,17] that AG and two L-arginine analogues, L-NNA and L-NAME, were equipotent in inhibiting the NO production induced by endotoxin only in rat aortic rings, suggesting that the role of AG may be due to incomplete inhibition of NOS, or that the development of immunological hepatic damage[18-21] may involve both NO-sensitive and NO-insensitive phases.

It has been recognized that the cNOS is Ca²⁺ insensitive phases. The present results showed that AG failed to inhibit completely the formation of cGMP by inflammatory stimuli, in contrast to NO production. Therefore, the effects of AG and two L-arginine analogs on the cGMP level were different, supporting the hypothesis that AG is a selective inhibitor for inducible isoform of NOS.

The specificity and possible mechanisms of...
various L-arginine analogs as NOS inhibitors have been investigated. Recent work suggested that L-NAME and L-NNA may affect NOS activity by modifying electron transfer through iron centers, and then limits arginine transport or utilization\[26,27\]. However, it is unlikely that a difference in the transport of AG into the cells can explain the lack of effects of this inhibitor on cNOS since it effectively inhibits iNOS\[28\]. Furthermore, the difference between in affinity or in arginine binding sites for the constitutive and inducible isoforms of NOS may not be used to explain the differences between the effects of L-arginine analogs and AG, since iNOS is not only regulated in the enzyme activity but also in the gene expression level\[29,30\]. Moreover, in the present study the results showed that AG and transcriptional inhibitor ActD were equipotent in decreasing NO production triggered by inflammatory stimuli in vitro, and both effects more potent than that induced by dexamethasone, the latter have non-specific and post-transcriptional inhibitory effect on NOS enzyme activity\[31-33\], suggesting that there is possibility of other mechanism in the AG inhibition, which in addition to competitive inhibition in the substrate level, also in gene transcription level. Further experiments will be required to determine whether AG directly affected the transcription of the iNOS gene or via the modulation of an intermediary protein(s) activity which indirectly influence on mRNA stability.

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