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Inhibition of Inducible Nitric Oxide Synthase Lowers the Cochlear Damage by Lipopolysaccharide in Guinea Pigs

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Endotoxin-treated cochleas of the guinea pig were examined electrophysiologically and immunohistochemically concerning the expression of inducible nitric oxide synthase (iNOS/NOS II). One mg of bacterial lipopolysaccharide (LPS, 5 mg/ml) or mixed solution of 1 mg of LPS plus 1 mg of N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg/ml) (L-NAME/LPS) was injected into the middle ear of guinea pigs transtympanically. The electrocochleograms were measured prior to, immediately and 48 h after the injection. Immunohistological studies for iNOS followed after fixation, embedding and sectioning of the temporal bones.

The threshold and amplitude of the compound action potential (CAP) became significantly worse in the LPS treated group. In contrast, the changes of the threshold and amplitude of CAP were decreased in the L-NAME/LPS group. iNOS was expressed in the stria vascularis, the spiral ligament, the organ of Corti and the spiral ganglion in the LPS group. These immunoreactivities in the L-NAME/LPS group were less intense than that in the LPS group. These results indicate that LPS has an ototoxic effect on the cochlea and that this effect could be mediated by iNOS produced high nitric oxide under inflammatory conditions.

Keywords: Inducible nitric oxide synthase, NOS-inhibitor, endotoxin, inner ear disturbance

INTRODUCTION

Nitric oxide (NO) possesses various biological activities. A small amount of NO is released under physiological conditions by constitutive NO synthase (NOS). In the cardiovascular system NO which is produced by endothelial NOS (eNOS/NOS III) acts as an endothelium-derived relaxing factor (EDRF). eNOS is Ca^{++} dependent and expressed by the stimulation of vasoconstrictory substances.[1–3] NO catalyzed by eNOS regulates the blood flow and pressure through relaxation of the smooth muscle cells of blood vessels. Brain NOS (bNOS/NOS I) is detected mainly in the central nervous system. bNOS activity is also observed in peripheral nerves and can influence the release of neurotransmitters.[1,4]

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On the other hand, increased NO levels can be observed under inflammatory conditions or after processing the tissue with inflammatory mediators, such as bacterial lipopolysaccharide (LPS), interferon-γ, interleukin-1 or tumor necrosis factor (TNF). The contact leads to the expression of inducible NOS (iNOS/NOS II) which is Ca^{++} independent. iNOS catalyzes large amounts of NO from L-arginine.[5] NO itself is a radical species and reacts with superoxide.

In this study a single dose of LPS or the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), or both simultaneously, were injected into the middle ear cavity of guinea pigs. LPS is known to penetrate the round window membrane,[6] cause dysfunction in the inner ear,[7] and induce the expression of iNOS.[8] Takumida *et al.*[9,10] reported that in the vestibular system iNOS activity is related to vestibular dysfunctions and these effects were blocked by L-NAME. However, the role of iNOS for the cochlear dysfunction remains unclear. Thus, we examined the effect of LPS and L-NAME by electrocochleography (ECochG) and immunohistochemistry and discussed the mechanism of the cochlear damage induced by iNOS under inflammatory conditions.

MATERIALS AND METHODS

Materials

Twenty-four guinea pigs weighing between 250 and 350 g were used in this study. All animals were confirmed to have a positive Preyer's reflex and be healthy otomicroscopically. Animals were anesthetized with 5% ketaminhydrochloride (50 mg/kg b.w.) and 2% xylazinhydrochloride (10 mg/kg b.w.).

The animals were divided into LPS, L-NAME plus LPS (L-NAME/LPS), L-NAME and control groups. In the LPS group ($n = 6$) 1 mg of LPS (Lot. 76H4100, Sigma, St. Louis, USA) dissolved in 0.2 ml saline (NaCl 0.9%) was injected into both middle ear cavities through the anterio-superior quadrant of the tympanic membrane. In the L-NAME/LPS group ($n = 6$) 1 mg of LPS and 1 mg of L-NAME (Lot. 37H0382, Sigma, St. Louis, USA) dissolved in 0.2 ml saline (NaCl 0.9%) were injected. In the L-NAME group ($n = 6$) 1 mg of L-NAME dissolved in 0.2 ml saline (NaCl 0.9%) was injected. Animals of the control group ($n = 6$) received 0.2 ml saline (NaCl 0.9%). This investigation was permitted by the Ethical Committee of Animal Experimentation (Bezirksregierung Köln/Germany, permit no.: 23.203.2 K42 3/98).

Immunohistochemical Examination

All animals were sacrificed after 48 h. The tissues were fixed via cardiac perfusion with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) after flushing out the blood with 0.1 M PBS. The cochleas were immersed in the same fixative overnight. Decalcification was performed with 10% EDTA solution in Tris (pH 7.0) for 5 days. Subsequently, the tissues were embedded into paraffin. Each specimen was sectioned at a thickness of 8 μm with a microtome (Microm, HM360, Germany). Deparaffinization was done by immersion in graded series of ethanol. Then the sections were immersed in 3% H_2O_2 for 20 min, followed by 0.25% Triton X for 10 min. Subsequently they were incubated with the first antibody to iNOS at 1 : 1000 dilution (rabbit polyclonal antibody, SA200, Biomol, Germany) overnight. After rinsing with 0.1% Tris–PBS solution (pH 7.6) and treated with 3% normal goat serum, the sections were incubated in the second antibody at 1 : 400 dilution (anti-rabbit, Dako, Denmark). The reaction was developed with a horse radish peroxidase (HRP) complex at 1 : 100 dilution for 1 h (Amersham Life Science, UK) and a nickel enhanced DAB (Sigma, St. Louis, USA).

ECochG Measurement

ECochG recordings were performed prior to, immediately and 48 h after the injection. The electrodes used were made of silver wire coated

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by polytetrafluorethylene and 0.25 mm in diameter (Goodfellow, Cambridge, UK). A small incision was made on the post-auricular portion, then, the bulla was exposed. One electrode was inserted into the facial canal through the stylomastoid foramen and pushed forward near to the round window (approximately 5 mm).[11–13] Dental cement was used to fix the electrode on the bony surface of the bulla. The indifferent and ground electrodes were placed on the top of the head and contralateral portion, respectively.

Acoustic stimuli were delivered by an earphone through a small tube inserted into the external ear meatus in a sound proof box. The stimuli were clicks at a rate of 11.1/s and a duration of 0.11 ms. Responses were accumulated 200 times by means of electrodiagnostic system (Nicolet Pathfinder 1, Nicolet Biomedical Instruments, Wisconsin, USA). The levels of stimuli were decreased from 95 dBSPL to 10 dBSPL by 5 dB steps. The compound action potential (CAP) amplitudes were calculated from the baseline to amplitude of N1. The CAP threshold was determined as the minimum sound level giving reproducible waveform. The recordings were repeated twice at the threshold level and the reproducibility was confirmed.

RESULTS

Expression of iNOS by Immunohistochemistry

In the L-NAME and control group, iNOS expression was not detected in the cochlear tissue (Figure 1(c)). In the LPS group, immunoreactivity for iNOS was detected after 48 h. iNOS activity was observed from basal turn to apex of cochlea. iNOS was expressed in the lateral wall, the supporting cells of the organ of Corti and the spiral ganglion cells (Figure 1(a)). In the organ of Corti, supporting cells (inner and outer pillar cells and Deiter's cells) exhibited iNOS immunoreactivity (Figure 1(f)). The inner and outer hair cells were not stained. In the lateral wall, the stria vascularis and the spiral ligament showed immunoreactivity for iNOS (Figure 1(d)). iNOS was also expressed in the perikarya of the ganglion cells (Figure 1(e)). These immunoreactivities were detected in the L-NAME/LPS group, however, less intense than that in the LPS group (Figure 1(b)).

Threshold Shifts of the CAP

The average threshold shifts of the CAP before, immediately and 48 h after the injection are presented in Figure 2(a). In all groups, threshold shifts of the CAP explainable by conductive hearing loss were observed after the injection into the middle ear. As proved by the control group, the differences of the average thresholds before and immediately after injection were not significant among all groups. The threshold shift of the CAP elevated significantly after 48 h only in the LPS group (ANOVA, $p < 0.001$). In the L-NAME and control group, the average threshold shifts tended to recover.

Comparing the threshold levels immediately after the injection to that 48 h after, the threshold shift was significant only in the LPS group (paired-*t*, $p < 0.0002$). In the remaining groups, including the L-NAME/LPS group, the threshold levels did not change significantly.

Changes of the CAP Amplitudes

The time course changes of the CAP amplitude at 95 dBSPL are presented in Figure 2(b). After a 48-h exposure to LPS the CAP amplitudes decreased significantly only in the LPS group (ANOVA, $p < 0.02$). The differences of the average amplitudes of the CAP before and immediately after the injection were not significant among all groups. There was a tendency to decrease the CAP amplitudes in the L-NAME/LPS group. On the other hand, the CAP amplitudes tended to increase in the L-NAME and control group.

Same as threshold shifts of the CAP, the differences of the average CAP amplitudes

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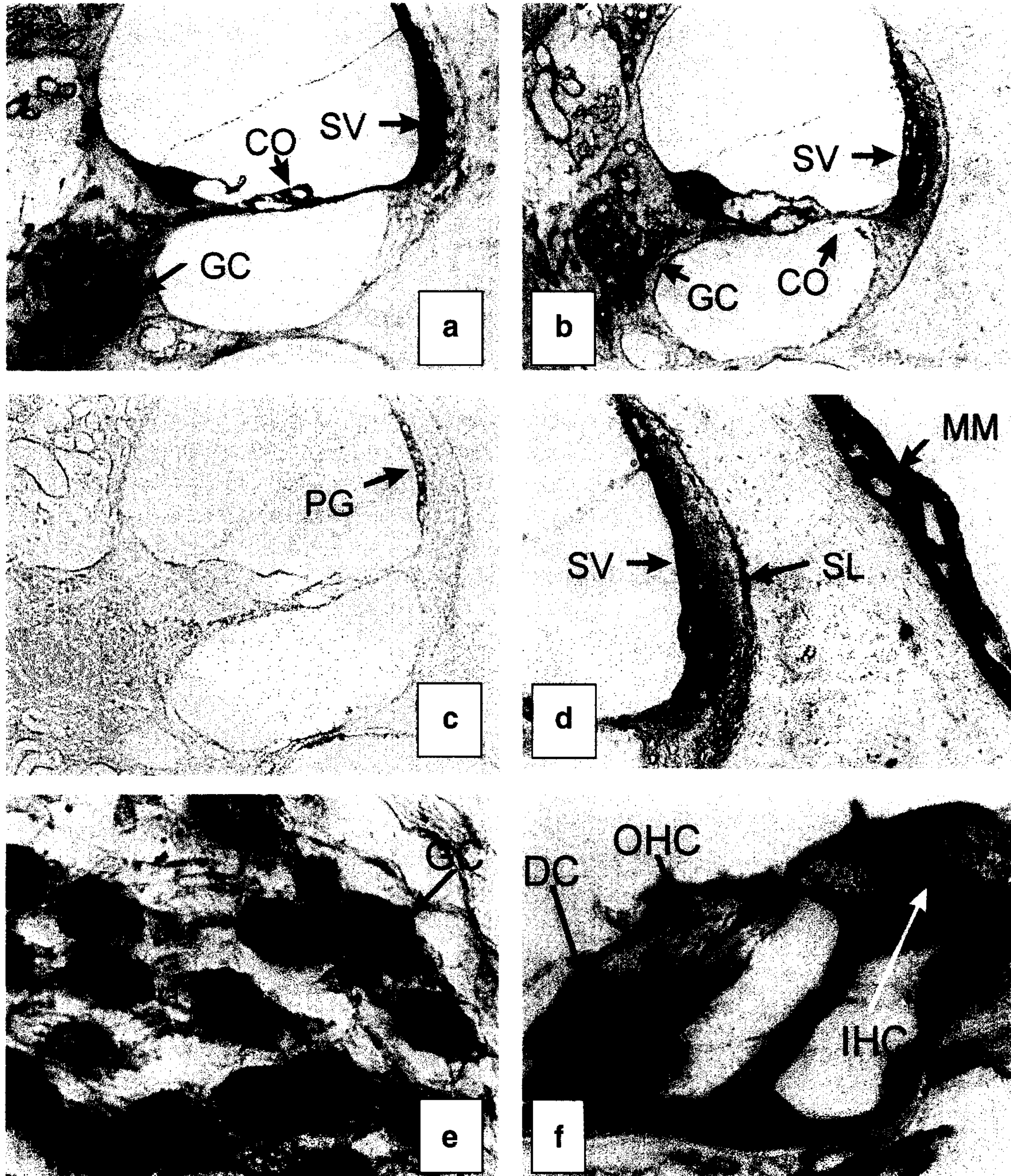

FIGURE 1 Paraffin sections of the cochlea, 8 μm. Immunohistochemistry, anti-iNOS. (a) LPS treated cochlea, iNOS is mainly expressed in the organ of Corti (CO), the stria vascularis (SV) and the spiral ganglion cells (GC), ×25. (b) L-NAME/LPS treated cochlea, iNOS is also positive in the organ of Corti, the stria vascularis and the spiral ganglion cells, however, these immunoreactivities were weaker than that in the LPS group, ×25. (c) Negative control, iNOS activity is not detected. Natural pigmentation (PG) of the stria vascularis is detected in the stria vascularis, ×25. (d) In the lateral wall, the spiral ligament (SL), the stria vascularis (SV) and the thickened mucosal membrane of the middle ear (MM) show reactivity, ×50. (e) The spiral ganglion cells (GC) have reactivity to iNOS, ×100. (f) The organ of Corti is shown. iNOS is positive in the supporting cells (Deiter's cells, DC). However, there is no apparent immunoreactivity in the sensory cells (inner and outer hair cells, IHC/OHC), ×250.

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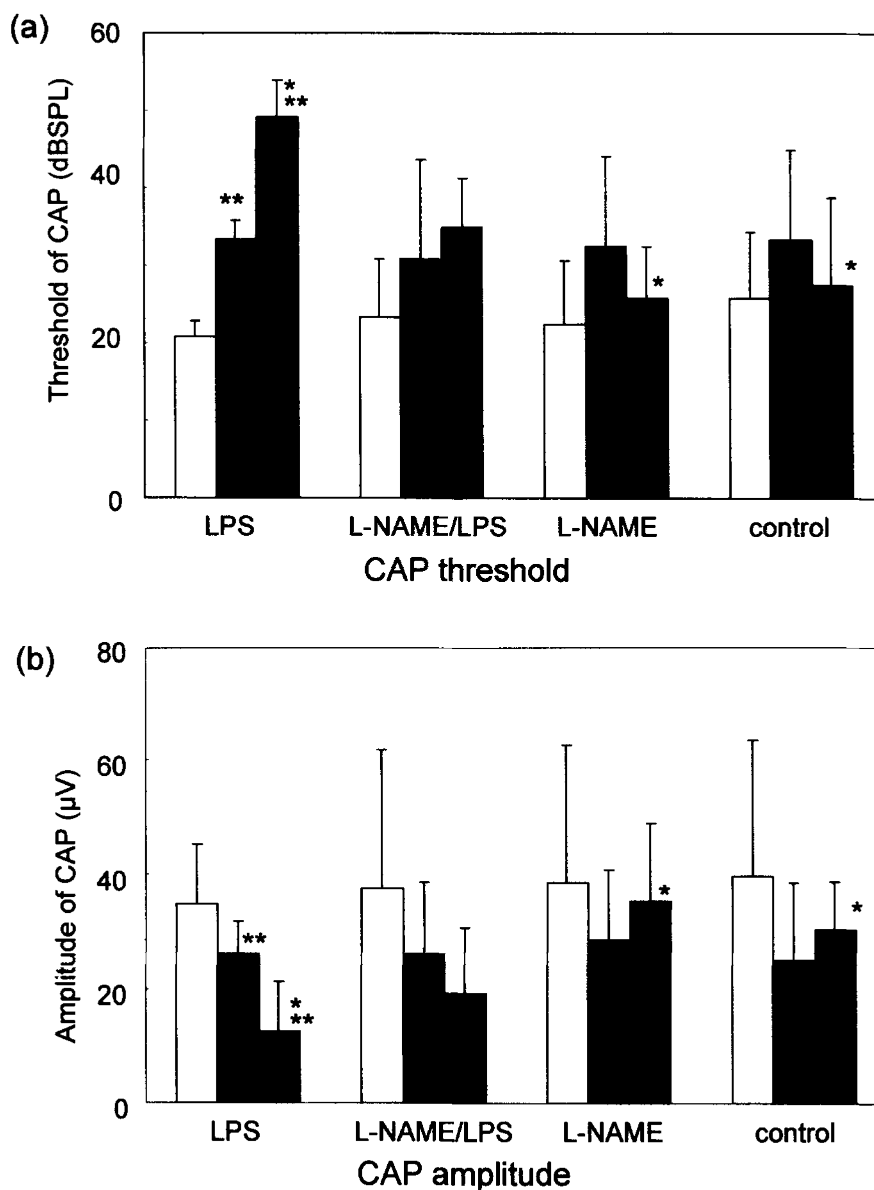

FIGURE 2 Changes of the CAP threshold and amplitudes. (a) Changes of the CAP threshold. The mean and 1.0 SD of the CAP threshold (white box; before, gray box; immediately after and black box; 48 h after the injection). In all groups, the threshold shifts which were explainable by the conductive hearing loss were observed. There were significant elevations of the CAP threshold after 48 h in the LPS group (ANOVA, $p < 0.001$*). This threshold shift was decreased in the L-NAME/LPS group. In the L-NAME and control groups, the threshold levels tended to recover. Comparing the threshold levels immediately after the injection to that 48 h after, the threshold shift was significant only in the LPS group (paired-t, $p < 0.0002$**). In the L-NAME/LPS group, the threshold level did not change significantly. (b) Changes of the CAP amplitudes. The mean and 1.0 SD of the CAP amplitudes at 95 dBSPL (white box; before, gray box; immediately after and black box; 48 h after the injection). There was a decrease of the CAP amplitude immediately after the injection of each solutions into the middle ear cavity same as the threshold levels. The CAP amplitudes decreased significantly after 48 h in the LPS group (ANOVA, $p < 0.02$*). In the L-NAME/LPS group, the changes of the amplitudes were less than that in the LPS group. In the L-NAME and control group there were slight increases of the CAP amplitudes. Same as threshold shifts of the CAP, the differences of the average CAP amplitudes immediately after the injection and 48 h after were examined. The CAP amplitudes decreased significantly only in the LPS group (paired-t, $p < 0.01$**). There were no significant changes in other groups.

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immediately after the injection and 48 h after were analyzed. The CAP amplitudes decreased significantly only in the LPS group (paired-*t*, $p < 0.01$). There were no significant changes in other groups.

DISCUSSION

The bacterial endotoxin LPS strongly induces inflammation and leads to inner ear malfunction.[7–10] Immunohistological study revealed that iNOS was expressed in the stria vascularis, spiral ligament, the organ of Corti (supporting cells) and spiral ganglion cells as previously reported.[14] NO in the circulatory system is thought to regulate the tone of cochlear blood vessels. An impairment of cochlear blood flow and permeability by increased levels of iNOS generated NO can be expected.[15,16] Kong *et al.*[17] reported that the exogenously applied NO changed the endocochlear potential which derives from the stria vascularis and increased the cochlear blood flow. This report supports that NO has a direct ototoxicity to the stria vascularis. NO also inhibits the cytochrome oxidase and mitochondrial respirations.[18,19] Inadequate amounts of NO produced by iNOS can lead an impaired homeostasis of the endolymphatic fluid.

Our electrophysiological data showed significant changes of the threshold and amplitude of the CAP in the LPS group after 48-h-exposure to endotoxin. In the L-NAME/LPS group, we could detect the threshold shifts and decrease of the CAP amplitude, however, they were more less than that in the LPS group. Whereas threshold levels and amplitudes of the CAP tended to recover in the L-NAME and control groups. These phenomena could be explained by the ventilation of the fluid in the middle ear cavity through the Eustachian tube.

L-NAME is a competitive and non-specific NOS inhibitor.[20] L-NAME suppresses both constitutive NOS (cNOS) and iNOS. Under physiological conditions, cNOS works to maintain the homeostasis of the inner ear. There is a possibility that L-NAME influences physiological conditions; for example, the administration of L-NAME causes reversible hypertension in normal animals because of inhibitory effect on eNOS.[21] However, we could detect no significant differences of the CAP threshold and amplitude between in the L-NAME and control group. L-NAME itself is supposed to have no detectable effect on the cochlea.[22] It has been reported that the cochlea is capable of iNOS expression after endotoxin treatment.[14] iNOS produces high level of NO under inflammatory conditions. NO is a free radical and reacts with superoxides which have direct cytotoxic effects. Dais *et al.*[23] showed that cochlear perfusion with sodium nitroprusside, a NO donor, caused significant elevations of the CAP threshold. Amaee *et al.*[24] reported that preperfusion of L-methyl arginine, an antagonist, protected the cochlea from the lesion mediated by sodium nitroprusside. On the basis of these reports, iNOS generated NO is thought to be responsible for the inner ear injury.

Immunohistochemical study revealed that in the L-NAME/LPS group showed less immunoreactivity for iNOS than that in the LPS group. Takumida *et al.*[25] pointed out that L-NAME blocks the production of NO and cytotoxicity of LPS. It is known that the damaged cells stimulate release of cytokines.[26,27] Some treatments, e.g. cytokines (interferon-γ, interleukin-1, tumor necrosis factor-α) or bacterial endotoxin (LPS) activate iNOS expression.[2,3,8,9] From these reports, it is supposed that LPS and cytokines may accelerate mutually the NO production and L-NAME blocks not only the production of NO but also indirectly the cytokines mediated pathway by reducing the cell damage (Figure 3).

In conclusion, our study demonstrates that LPS treatment induces iNOS expression and functional change on the cochlea and NOS inhibitor lowered the immunoreactivities for iNOS and hearing loss. Thus, iNOS mediated NO and free radical species are likely to mediate cochlear malfunction under inflammatory conditions.

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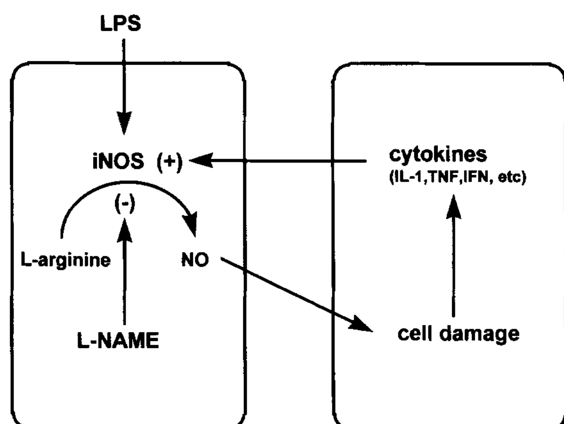

FIGURE 3 Schematic drawing of iNOS mediated cell damage. iNOS mediated high amounts of NO are cytotoxic. Damaged cells stimulate release of cytokines. These cytokines, e.g. IFN-γ, IL-1 or TNF-α, activate the expression of iNOS. L-NAME is supposed to block not only the production of NO but also indirectly the cytokines mediated pathway by lowering cell damage.

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