ORIGINAL ARTICLE

Expression of Inducible Nitric Oxide Synthase in a Mouse Model of Anaphylaxis

K Sade,[1,2] IF Schwartz,[2,3] S Etkin,[1] S Schwartzenberg,[2] Y Levo,[2] S Kivity[1]

[1] The Allergy & Asthma Center, Tel-Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel
[2] Department of Medicine 'T', Tel-Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel
[3] Department of Nephrology, Tel-Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

■ **Abstract**

Background: The generation of large quantities of nitric oxide (NO) is implicated in the pathogenesis of anaphylactic shock. The source of NO, however, has not been established and conflicting results have been obtained when investigators have tried to inhibit its production in anaphylaxis.
Objective: The aim of this study was to analyze the expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in a mouse model of anaphylaxis.
Methods: BALB/c mice were sensitized and challenged with ovalbumin to induce anaphylaxis. Tissues were removed from the heart and lungs, and blood was drawn at different time points during the first 48 hours after induction of anaphylaxis. The Griess assay was used to measure nitric oxide generation. Nitric oxide synthase expression was examined by reverse transcriptase polymerase chain reaction and immunohistochemistry.
Results: A significant increase in iNOS mRNA expression and nitric oxide production was evident as early as 10 to 30 minutes after allergen challenge in both heart and lungs. In contrast, expression of eNOS mRNA was not altered during the course of the experiment.
Conclusion: Our results support involvement of iNOS in the immediate physiological response of anaphylaxis.

Key words: Anaphylaxis. Shock. Allergy. Nitric oxide. Inducible nitric oxide synthase.

■ **Resumen**

Antecedentes: La producción de grandes cantidades de óxido nítrico (NO) se relaciona con la patogenia del choque anafiláctico. No obstante, el origen del NO no se ha establecido y los investigadores han obtenido resultados contradictorios al intentar inhibir su producción en la anafilaxia.
Objetivo: El objetivo de este estudio fue analizar la expresión de la óxido nítrico sintetasa inducible (iNOS) y de la óxido nítrico sintetasa endotelial (eNOS) en un modelo con ratón de la anafilaxia.
Métodos: Se sensibilizaron ratones BALB/c y se provocaron con ovoalbúmina para inducir la reacción anafiláctica. Se extrajeron tejidos de corazón y pulmones, así como sangre en distintos momentos durante las primeras 48 horas posteriores a la inducción de la reacción anafiláctica. Para medir la producción de óxido nítrico se usó la reacción de Griess. La expresión de la óxido nítrico sintetasa se examinó mediante retrotranscriptasa y reacción en cadena de la polimerasa e inmunohistoquímica.
Resultados: Se produjo un aumento significativo en la expresión del ARNm de la iNOS en corazón y pulmones entre 10 y 30 minutos después de la provocación con alérgenos. Contrariamente, la expresión del ARNm de la eNOS no se vio alterada en el transcurso del experimento.
Conclusión: Nuestros resultados confirman que la iNOS está involucrada en la respuesta fisiológica inmediata de la anafilaxia.

Palabras clave: Anafilaxia. Choque. Alergia. Óxido nítrico. Óxido nítrico sintetasa inducible.

Introduction

Anaphylaxis is a life-threatening systemic allergic response that can lead to multisystem failure, shock, and death. Its rapid onset and its unpredictable course complicate research into this phenomenon in both humans and animals. Causes of anaphylaxis include bee stings, medications, food, latex exposure, and exercise. It is not as rare as generally believed and may affect as much as 1.2% to 15% of the population. Consequently, treatment is still unsatisfactory and the mortality rate is unacceptably high [1].

The main mechanism underlying anaphylaxis involves a sudden release of mast cell-derived and basophil-derived mediators, including histamine, tryptase, serotonin, platelet-activating factor, leukotrienes, prostaglandins, cytokines, and nitric oxide (NO). The role of each of these mediators in the pathogenesis of anaphylactic shock is still not defined [1,2]. The development of symptoms and signs depends on the nature of the allergen, its quantity, and its route of administration. Hypotension, the most worrisome event, can develop rapidly and may be followed by protracted shock [1].

Evidence accumulated over the past decade has suggested that increased production of NO mediates the protracted hypotension associated with septic and other forms of shock, including anaphylaxis [2-4]. This hypothesis was supported by the observation of high levels of NO in cases of anaphylactoid or anaphylactic reactions in both humans and animals [4-9]. The source of NO, however, was not established and conflicting results were obtained when investigators tried to inhibit its production in anaphylaxis [9-15].

Molecular cloning and sequence analysis revealed the existence of 3 distinct isoforms of nitric oxide synthase (NOS) that differ in activity and tissue distribution. Brain NOS and endothelial NOS (eNOS) are constitutively expressed and are tightly regulated in order to produce only small amounts of NO. In contrast, inducible NOS (iNOS) is regulated mainly at the transcriptional level, but once induced by cytokines or endotoxins, it produces large amounts of NO for long periods of time [16,17]. iNOS was found to be the cause of the high output of NO in inflammatory conditions such as sepsis, rheumatoid arthritis, inflammatory bowel disease, and allergic reactions, including asthma [16-18]. In contrast to these inflammatory conditions, anaphylaxis is the clinical manifestation of an immediate allergic reaction that occurs long before inflammation takes place. This adverse event is characterized by a rapid and often dramatic onset, with a sharp rise in NO concentration [9-15]

Since iNOS is induced and regulated mainly at the transcriptional level, its activation depends on de novo synthesis of both RNA and protein. Therefore, it was assumed that the induction of iNOS requires a lag period of at least 4 to 8 hours. In anaphylaxis, however, the hemodynamic response to allergen has been documented as early as a few minutes following challenge, implying that constitutively expressed NOS may be responsible for the hemodynamic response [7-12,19,20].

Using a murine model of sepsis, we have previously shown that rapid activation of iNOS is responsible, at least in part, for the hemodynamic changes in sepsis and that iNOS mRNA expression can be demonstrated as early as 60 minutes after endotoxin administration [21]. Although the time course of expression of iNOS or other NOS has not been directly investigated in allergic reactions, we recently found evidence that iNOS is expressed 3 hours after allergen challenge in a murine model of acute asthma, before chronic inflammatory or structural changes of persistent asthma occurred [21]. In view of these findings, we designed the current experiments to determine the time course of iNOS and eNOS expression in a murine model of anaphylaxis. Using reverse transcriptase polymerase chain reaction (RT-PCR) and the Griess reaction, we analyzed the expression of NOS isoforms and serum concentrations of nitrates/nitrites (the stable products of NO generation), respectively.

Materials and Methods

Animals

All experiments were performed in accordance with recommendations on the care and use of laboratory animals and were approved by the local ethics committee for animal use. Female BALB/c mice, 6 to 8 weeks of age, were maintained on an ovalbumin (OVA)-free diet with food and water provided ad libitum.

OVA Sensitization and Challenge

All the mice were sensitized on days 1 and 14 by intraperitoneal injection of 100 µg OVA (Grade V; Sigma Chemical Co, Rehovot, Israel) emulsified in 8 mg aluminum hydroxide (Sigma Chemical Co) in a volume of 100 µL. Anaphylaxis was induced on day 28 by a single intraperitoneal injection of 0.2 mL OVA 100 µg/µL. At designated time points (10 minutes, 30 minutes, and 1, 2, 4, 16, 48, and 72 hours after challenge), blood was drawn from the orbital sinus or plexus of the mouse. The animals were sacrificed and the heart and lungs were removed for further analysis.

Intradermal Skin Testing

To confirm the validity of the sensitization protocol, 5 randomly chosen mice were tested for immediate cutaneous hypersensitivity reactions using an intradermal skin test 1 week after a second sensitization with OVA. The skin of the belly was shaved under light anesthesia with sodium brevital (10 mg/dL). For each skin test, 20 µL OVA diluted at 500 µg/mL in phosphate buffered saline (PBS) was injected intradermally with a 30-gauge needle while the skin was stretched taut. An injection of saline was used as a negative control and histamine was used as a positive control. The wheal reactions were assessed 20 minutes after the injection. A reaction was defined as positive if the wheal diameter was greater than 3 mm in any direction.

Assessment of Anaphylaxis

Signs of allergic reaction were evaluated by using a scoring system as follows: 0 = no signs; 1 = scratching and rubbing around the nose; 2 = increased respiratory rate; 3 = labored inspiration or cyanosis around the mouth, tongue, or tail; 4 = no activity or convulsions; 5 = death.

Measurement of Plasma Histamine Concentration

To determine plasma histamine concentration, blood was collected after allergen challenge at the designated time points. Serum was prepared and stored at –80°C until analyzed. Histamine content was determined by enzymatic isotopic assay as described by Beaven et al [22].

Nitrite Assay

Nitrites were analyzed by adding 50 µL freshly prepared Griess reagent (1% sulfanilic acid in 5% H_3PO_4 and 0.1% N-1-naphthyl ethylene diamine dihydrochloride) in double-distilled H_2O to 50 µL of serum from each experimental group. Samples were incubated at room temperature for 20 minutes and spectrophotometric readings were taken at 546 nm. The nitrite concentrations were determined using a linear standard curve between 1 and 150 µmol/L sodium nitrite. Assays were performed in triplicate [23].

Nitrate Assay

Nitrate content in the serum was measured by bioassay using a modified version of Bartholomew's method [24]. Nitrate was reduced to nitrite with nitrate reductase obtained from *Escherichia coli*. The bacteria were grown for 18 hours under anaerobic conditions after being washed, resuspended in PBS, and stored at –70°C until use. Nitrates were then measured with the Griess reagent as described previously [24].

Detection of iNOS and eNOS mRNA

Total mRNA was extracted from all isolated tissues. For RT-PCR analysis, 10 µg RNA was reverse transcribed with 10 µL of 5× buffer, 5 µL dithiothreitol (5 mmol/L), 10 µL deoxyribonucleoside triphosphate (2.5 mmol/L), 0.7 µg oligo-dT, and 1 µg of reverse transcriptase in a total volume of 50 µL. The reaction was terminated by heating the samples at 65°C for 10 minutes. PCR amplification was performed in a reaction volume of 100 µL with a thermal cycler. Ten microliters of cDNA was added to a reaction mixture containing 10 µL of 10× buffer, 2.5 µL deoxyribonucleoside triphosphate (2.5 mmol/L), and 100 pmol of each of the oligonucleotide primers in the presence of 0.5 µL Taq DNA polymerase (1.25 units).

The primers used for iNOS were 5´-GTG TTC CAC CAG GAG ATG TTG-3´ (sense) and 5´-CTC CTG CCC ACT GAG TTC GTC-3´ (antisense). The primers used for eNOS were 5'-CCG GAA TTC GAA TAC CAG CCT GAT CCA TGG AA-3' (sense) and 5'-GCC GGA TCC TCC AGG AGG GTG TCC ACC GCA TG-3' (antisense) [25, 26].

Thirty-five cycles were performed with the following PCR conditions: 95°C for 1 minute (denaturation), 54°C for 1 minute (annealing), 72°C for 2 minutes (extension), and 72°C for 7 minutes (final extension). Fifteen microliters of PCR products was analyzed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. The conditions for iNOS and for eNOS RT-PCR were similar.

To assess the adequacy of the cDNA and the efficiency of the RT-PCR system, murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as RT-PCR controls under the same conditions. The cDNA of GAPDH was amplified with the following primers: 5´-AAT GCA TCC TGC ACC ACC AA-3´ (sense) and 5´-GTA GCC ATA TTC ATT GTC ATA-3´ (antisense) [25,26]. A 100-bp DNA ladder was used to identity the molecular weight of the target DNA. Stained gels were photographed and the films were scanned. The intensities of eNOS, iNOS, and GAPDH bands were calculated and the steady-state mRNA levels were expressed in arbitrary units as the ratio of iNOS or eNOS to GAPDH expression.

Immunohistochemistry

The expression of iNOS protein in lungs was determined by immunohistochemistry. Lung samples were immediately fixed in 4% formaldehyde (w/v) in PBS and embedded in paraffin. Immunohistochemistry was performed with a polyclonal antibody for iNOS detection (Santa Cruz, CA, USA). Sections (3 µm) were mounted on Super Frost Plus glass (Menzel-Glazer, Braunschweig, Germany) and processed in an automated immunostainer (Ventana-ES, Ventana Medical System, Tucson, USA).

After deparaffinization, immunohistochemistry was performed using a 3-step indirect process based on the labeled avidin-biotin peroxidase complex method. The sections were incubated for 32 minutes with a 1:50 dilution of iNOS antibodies. Immunohistochemistry was performed using the I-View DAB detection kit (Ventana Medical System) according to the manufacturer's instructions. The I-View DAB detection kit utilized biotinylated secondary antibodies followed by streptavidin–horseradish peroxidase conjugate. The complex was then visualized with hydrogen peroxide substrate and 3,3'-diaminobenzidine tetrahydrochloride chromogen, which produces a dark brown precipitate that is readily detected by light microscopy. The incubations were performed at a controlled temperature of 42°C. The sections were then counterstained with Gill's hematoxylin, dehydrated, and mounted for microscopic examination.

Allveolar, bronchial, and vascular iNOS staining intensity was assessed semiquantitatively on coded material by light microscopy and expressed as follows: 0, absence of staining; 1, light staining; 2, moderate staining; 3, intense staining. The scoring was determined twice by 2 observers who were unaware of the timing of animal sacrifice.

Statistical Analysis

Values are expressed as the mean (SEM). Data from the various experiments were subjected to analysis of variance and $P < .05$ was considered statistically significant.

Results

Sensitized mice developed anaphylaxis as early as 10 minutes following allergen challenge and the clinical score peaked at 30 minutes. The overall mortality rate was 25%: 70% of the mortalities occurred during the first 4 hours and 30% occurred between 4 and 16 hours after induction of anaphylaxis. Mice that survived after 16 hours did not show any signs of disease (Figures 1 and 2). In parallel with the clinical score, blood histamine levels rose rapidly, peaked at 30 minutes, and then returned to baseline (Figure 3).

iNOS and eNOS mRNA Expression

The PCR products amplified with iNOS and eNOS primers showed clear bands at the predicted size. These bands were absent in the products obtained in PCR reactions from RNA processed without reverse transcriptase, indicating that they originated from mRNA and not from genomic DNA. The GAPDH signal was the same in all samples, indicating that the effect of allergen challenge on mRNA expression was specific and, consequently, that GAPDH could be used as an internal standard for comparing the intensities of RT-PCR products for iNOS and eNOS.

An increase in iNOS mRNA expression was evident in the lung and heart as early as 10 minutes after allergen challenge. The intensity of iNOS mRNA expression reached its peak between 30 and 60 minutes after challenge and declined to baseline level after 4 hours. Sixteen hours after challenge, lung and heart iNOS
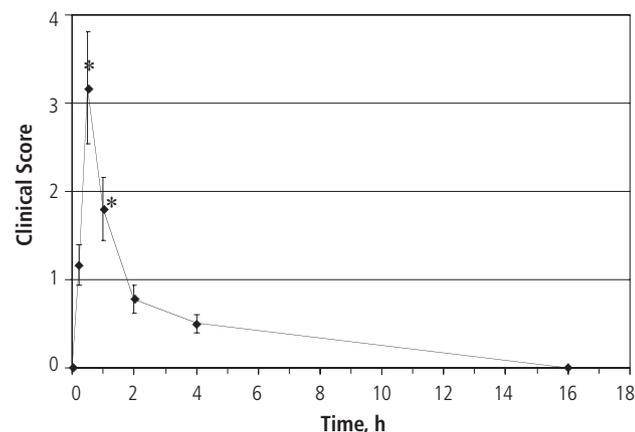
Figure 2. Mortality rates at different time points during anaphylaxis. Data are shown as the mean of 4 different experiments (n = 4 for each time point of every experiment) and whiskers indicate SEM.

Figure 3. Time course of serum histamine concentration during mouse anaphylaxis after in vivo administration of allergen. Data are shown as the mean of 4 different experiments (n = 4 for each time point of every experiment) and whiskers indicate SEM.
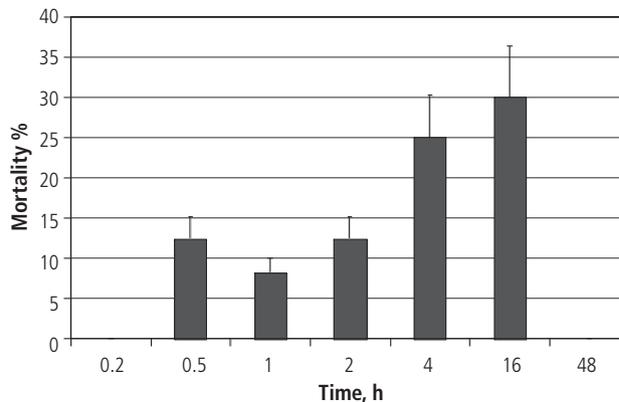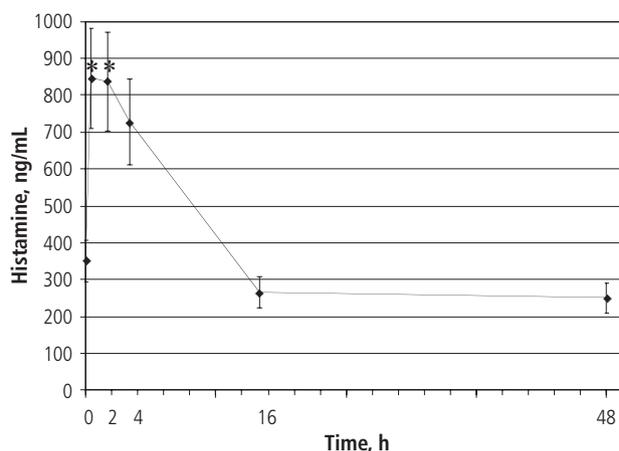 *$P < .05$ as compared with baseline (0 hours).

mRNA expression again increased before returning to baseline levels.

When the relative amount of iNOS PCR products was quantitated by densitometry and expressed as the iNOS/GAPDH ratio, the increase in iNOS expression was statistically significant between 30 and 60 minutes after OVA administration and again at 16 hours compared with baseline values (Figure 4). The time course of iNOS expression was the same for both the heart and the lung.

eNOS mRNA expression was low at baseline and remained unchanged throughout the entire experiment in both organs.

iNOS Immunostaining

To further establish whether iNOS mRNA expression accompanied iNOS protein expression, the expression of

Figure 1. Time course of clinical score for anaphylaxis after in vivo administration of allergen. Data are shown as the mean of 4 different experiments (n = 4 for each time point of every experiment) and whiskers indicate SEM.
*$P < .05$ as compared with baseline (0 hours).

A

0 0.5 2 4 16 48 h

576 bp
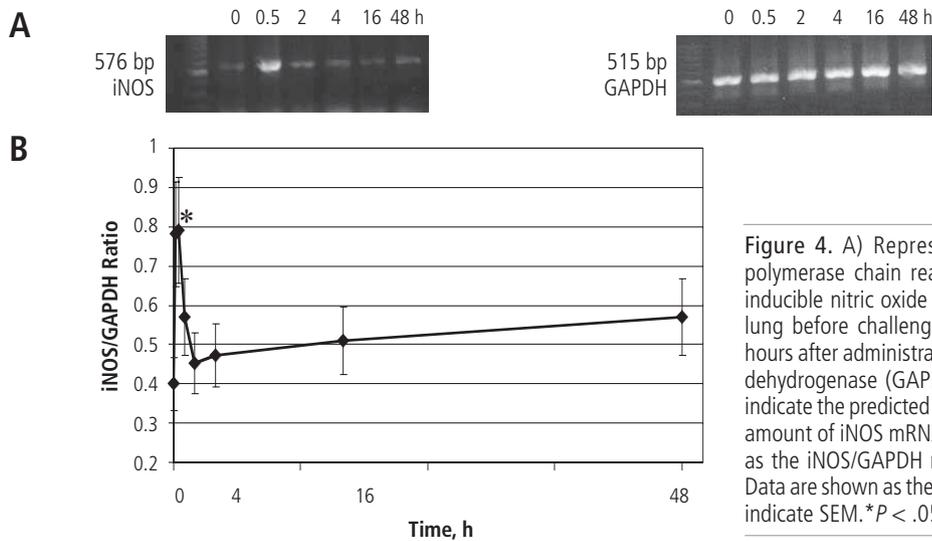iNOS

515 bp
GAPDH

0 0.5 2 4 16 48 h

B

Figure 4. A) Representative gels from reverse transcriptase polymerase chain reaction experiments showing time course of inducible nitric oxide synthase (iNOS) mRNA expression in mouse lung before challenge (0 hours) and 0.5, 1,2, 3, 4, 16, and 48 hours after administration of allergen. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as the internal control. Numbers indicate the predicted size of the amplified product. B) Mean relative amount of iNOS mRNA quantitated by densitometry and expressed as the iNOS/GAPDH ratio of the same experiments shown in (A). Data are shown as the mean of 3 different experiments and whiskers indicate SEM.*$P < .05$ as compared with baseline.
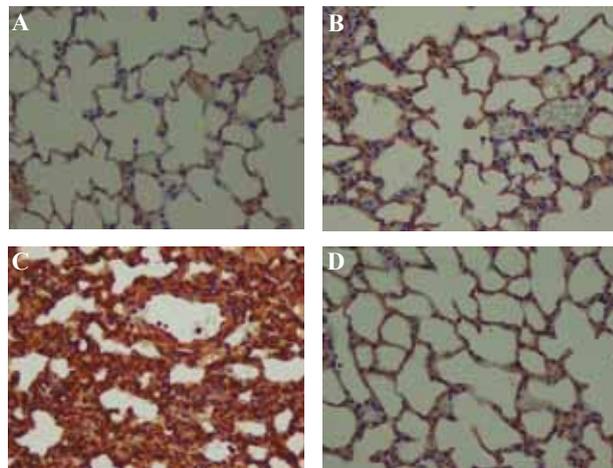

Figure 5. Immunohistochemical staining for inducible nitric oxide synthase in 4 μm sections of formalin-fixed, paraffin-embedded lung samples from ovalbumin-sensitized mice. Samples were taken before challenge (A) and 30 minutes (B), 4 hours (C), and 48 hours (D) after ovalbumin challenge. Original magnification, ×200.

Figure 6. Time course of serum $NO_2 + NO_3$ (NOx) concentration in anaphylactic mice after in vivo administration of allergen. Data are shown as the mean of 4 different experiments (n = 4 for each time point of every experiment) and whiskers indicate SEM.
*$P < .05$ as compared with baseline (0 hours).

iNOS was examined by immunohistochemistry on lung tissue taken from the mice at different time points. The intensity of the staining for iNOS in the lung epithelium was significantly higher 30 minutes after allergen challange (mean [SEM] score, 2.1 [0.18]) in comparison with baseline (1.3 [0.15]) ($P = .009$; Figure 5).

NOx Production

To determine whether changes in iNOS mRNA expression were accompanied by changes in enzyme activity, we analyzed systemic NO production during anaphylaxis. The mean baseline (ie, before OVA challenge) plasma concentration of the stable metabolites of NO, nitrates and nitrites (NOx), was
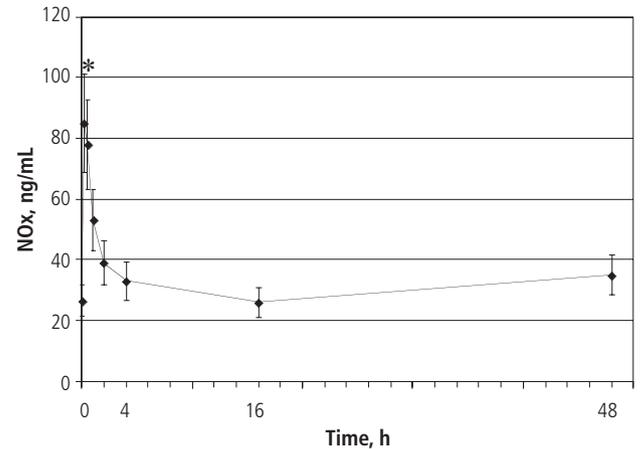
26.6 (0.8) μmol/L. These values increased to 84.9 (1.7) μmol/L ($P < .005$), 78 (1.9) μmol/L ($P < .005$), and 53.1 (3.8) μmol/L ($P < .05$) at 15, 30, and 60 minutes, respectively, after OVA administration, and then gradually decreased. No increase in plasma NOx concentration compared with baseline levels was detected at 16 hours after administration of OVA (Figure 6).

Discussion

The current set of experiments in a murine model of anaphylaxis provides evidence that there is an increase in expression of iNOS mRNA in both the lung and heart within a very short period of time after exposure to allergen. This increase was accompanied by histamine release and

clinical manifestations of an immediate allergic response. iNOS expression gradually declined thereafter and returned to baseline 48 to 72 hours after anaphylaxis. In contrast, expression of eNOS mRNA was not altered throughout the experiment.

Establishing the source of NO in anaphylaxis may help to elucidate its pathogenic role and lead to better therapeutic options. NO has dual effects in many pathological conditions, and this dichotomy is probably dependent on its source, concentration, and site of action. In sepsis, high levels of NO produced by iNOS lead to inhibition of eNOS, possibly via NO autoinhibition, thereby abolishing the protective effects of eNOS [27,28]. Recently, a similar situation was suggested for allergic reactions, such as asthma, where low levels of NO produced by constitutive eNOS were shown to play a positive role in regulation and protection of both the airways and vascular tone [29,30]. Any attempt to prevent anaphylactic shock by inhibiting NO production could fail if we do not take into account that this inhibition might also affect beneficial NO production in blood vessels or airways. In fact, inhibition of NO production with a nonspecific NOS inhibitor attenuated the reduction in blood pressure seen in anaphylactic shock but at the same time aggravated cardiac function, promoted bronchospasm, and worsened survival [10-15]. Mitshuata et al [14,15] showed a significant decrease in coronary blood flow in animals treated with an eNOS inhibitor, demonstrating the importance of eNOS in maintaining vascular tone and blood flow to vital organs during shock. Bellou et al [10] investigated the effect of an eNOS inhibitor on OVA-induced murine anaphylaxis and found a reduction of hypotension in rats pretreated with an NO synthase inhibitor only when it was associated with histamine receptor and serotonin receptor antagonists. This effect was transient, however, and the survival time did not increase. Recently, Cauwels et al [31] found a new and surprising role of eNOS in the pathogenesis of anaphylactic shock, and they suggested that eNOS-derived NO might be the principal vasodilator in shock. This concept is new and unexpected, and its implications are still unclear.

Our results support a pathogenic role for NO in anaphylaxis and suggest a new direction to investigate its mechanism of action. In accordance with our results, NO was found by others to be related to some aspects of late allergic reactions in various conditions and models, and in addition, it was considered a marker of the inflammatory component of asthma [32]. It is possible that the late expression of iNOS is one of the markers or even one of the causes of the late anaphylactic reaction and inflammation that occur after the initial immunoglobulin E-mediated allergic reaction.

We have still not identified the trigger for the induction of iNOS in anaphylaxis, nor do we know whether it is induced by cross-linking of the allergen itself or by secondary mediators. Several studies have demonstrated that platelet-activating factor is a major mediator of anaphylaxis, even in mast cell-deficient mice, and evidence has been accumulating to suggest that platelet-activating factor causes a rapid biphasic induction of nuclear factor (NF)-κB. [33,34] Since NF-κB is known as the main promotor of iNOS induction [33,34], we speculate that this might be the mechanism by which iNOS is induced in anaphylaxis.

Our finding that iNOS is rapidly induced in anaphylaxis highlights its importance in the pathogenesis of anaphylactic shock. It is tempting to speculate that using selective iNOS inhibitors will prevent anaphylactic vasodilatation and shock without inhibiting the protective effects of eNOS, but confirmation of this possibility awaits further study.

Acknowledgments

References

1. Kemp SF, Lockey RF. Anaphylaxis: A review of causes and mechanisms. J Allergy Clin Immunol. 2002;110:341-8.
2. Osada S, Ichiki H, Oku H, Ishiguro K, Kunitomo M, Semma M. Participation of nitric oxide in mouse anaphylactic hypotension. Eur J Pharmacol. 1994;252:347-50.
3. Szabo C, Thiemermann C. Invited opinion: role of nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury. Shock. 1994;2:145-55.
4. Rolla G, Nebiolo F, Guida G, Heffler E, Bommarito L, Bergia R. Level of exhaled nitric oxide during human anaphylaxis. Ann Allergy Asthma Immunol. 2006;97(2):264-5.
5. Viaro F, Dalio MB, Evora PR. Catastrophic cardiovascular adverse reactions to protamine are nitric oxide/cyclic guanosine monophosphate dependent and endothelium mediated: should methylene blue be the treatment of choice? Chest. 2002;122:1061-6.
6. Tsuda A, Tanaka KA, Huraux C, Szlam F, Sato N, Yamaguchi K, Levy JH. The in vitro reversal of histamine-induced vasodilation in the human internal mammary artery. Anesth Analg. 2001;93:1453-9.
7. Mitsuhata H, Saitoh J, Takeuchi H, Hasome N, Horiguchi Y, Shimizu R. Production of nitric oxide in anaphylaxis in rabbits. Shock. 1994;2:381-4.
8. Amir S, English AM. An inhibitor of nitric oxide production, NG-nitro-L-arginine-methyl ester, improves survival in anaphylactic shock. Eur J Pharmacol. 1991;203:125-7.
9. Abend Y, Ashkenazy Y, Witzling V, Feigl D, Geltner D, Moshonov S, Zor U. Nitric oxide: a mediator in anaphylactic shock in guinea-pigs. J Basic Clin Physiol Pharmacol. 1996;7:57-69.
10. Bellou A, Lambert H, Gillois P, Montemont C, Gerard P, Vauthier E, Sainte-Laudy J, Longrois D, Gueant JL, Mallie JP. Constitutive nitric oxide synthase inhibition combined with histamine and serotonin receptor blockade improves the initial ovalbumin-induced arterial hypotension but decreases the survival time in brown norway rats anaphylactic shock. Shock. 2003;19:71-8.
11. Fargeas MJ, Theodorou V, Weirich B, Fioramonti J, Bueno L. Decrease in sensitisation rate and intestinal anaphylactic response after nitric oxide synthase inhibition in a food hypersensitivity model. Gut. 1996;38:598-602.
12. Pallapies D, Thelen K, Dembinska-Kiec A, Simmet T, Peskar BA. Effect of 3-morpholinosydnonimine (SIN-1), NG-nitro-L-arginine (NNA) and NG-monomethyl-L-arginine (NMMA) on isolated anaphylactic guinea-pig hearts. Agents Actions Suppl. 1992;37:114-9.

13. Masini E, Gambassi F, Di Bello MG, Mugnai L, Raspanti S, Mannaioni PF. Nitric oxide modulates cardiac and mast cell anaphylaxis. Agents Actions. 1994;41 Spec No:C89-90.

14. Mitsuhata H, Takeuchi H, Saitoh J, Hasome N, Horiguchi Y, Shimizu R. An inhibitor of nitric oxide synthase, N omega-nitro-L-arginine-methyl ester, attenuates hypotension but does not improve cardiac depression in anaphylaxis in dogs. Shock. 1995;3:447-53; discussion 454.

15. Mitsuhata H, Saitoh J, Hasome N, Takeuchi H, Horiguchi Y, Shimizu R. Nitric oxide synthase inhibition is detrimental to cardiac function and promotes bronchospasm in anaphylaxis in rabbits. Shock. 1995;4:143-8.

16. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329:2002-12.

17. Nathan C. Inducible nitric oxide synthase: what difference does it make? J Clin Invest. 1997;100:2417-23.

18. Sade K, Schwartz I, Schwartz D, Wolman Y, Chernichovski T, Fireman E, Iaina A, Kivity S. Effect of montelukast pretreatment on inducible nitric oxide synthase mRNA expression in the lungs of antigen-challenged allergic mice. Clin Exp Allergy. 2003;33:1741-6.

19. Mitsuhata H, Shimizu R, Yokoyama MM. Role of nitric oxide in anaphylactic shock. J Clin Immunol. 1995;15:277-83.

20. Saitoh J, Mitsuhata H, Takeuchi H, Hasome N, Shimizu R. In vivo production of nitric oxide in the canine heart in IgE-mediated anaphylaxis. Shock. 1996;6:66-70.

21. Sade K, Schwartz D, Wolman Y, Schwartz I, Chernichovski T, Blum M, Brazowski E, Keynan S, Raz I, Blantz RC, Iaina A. Time course of lipopolysaccharide-induced nitric oxide synthase mRNA expression in rat glomeruli. J Lab Clin Med. 1999;134:471-7.

22. Beaven MA, Jacobsen S, Horakova Z. Modification of the enzymatic isotopic assay of histamine and its application to measurement of histamine in tissues, serum and urine. Clin Chim Acta. 1972;37:91-103.

23. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate and [15N] nitrate in biological fluids. Anal Biochem. 1982;126:131-8.

24. Bartholomew B. A rapid method for the assay of nitrate in urine using the nitrate reductase enzyme of Escherichia coli. Food Chem Toxicol. 1984;22:541-3.

25. Morrissey JJ, McCracken R, Kanteto H, Vehaskari M, Montani D, Klahr S. Location of an inducible nitric oxide synthase mRNA in the normal kidney. Kidney Int. 1994;45:998-1005.

26. Terada Y, Tomita K, Nonoguchi H, Marumo F. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. J Clin Invest. 1992;90:659-65.

27. Schwartz D, Mendonca M, Schwartz I, Xia Y, Satriano J, Wilson CB, Blantz RC. Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. J Clin Invest. 1997;100:439-48.

28. Hallemeesch MM, Janssen BJ, de Jonge WJ, Soeters PB, Lamers WH, Deutz NE. NO production by cNOS and iNOS reflects blood pressure changes in LPS-challenged mice. Am J Physiol Endocrinol Metab. 2003;285:E871-5.

29. Ricciardolo FL, Geppetti P, Mistretta A, Nadel JA, Sapienza MA, Bellofiore S, Di Maria GU. Randomised double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. Lancet. 1996;348:374-7.

30. Ricciardolo FL, Timmers MC, Geppetti P, van Schadewijk A, Brahim JJ, Sont JK, de Gouw HW, Hiemstra PS, van Krieken JH, Sterk PJ. Allergen-induced impairment of bronchoprotective nitric oxide synthesis in asthma. J Allergy Clin Immunol. 2001;108:198-204.

31. Cauwels A, Janssen B, Buys E, Sips P, Brouckaert P. Anaphylactic shock depends on PI3K and eNOS-derived NO. Clin Invest. 2006 Aug;116(8):2244-51.

32. Musoh K, Nakamura N, Ueda Y, Inagaki N, Nagai H. Possible role of nitric oxide in IgE-mediated allergic cutaneous reaction in mice. Int Arch Allergy Immunol. 1998;115:91-6.

33. Carvalho Tavares J, Bayon Y, Sanchez Crespo M. Immunoglobulin E-mediated anaphylaxis activates nuclear factor kappaB in rat small intestine. Inflamm Res. 1998;47:265-9.

34. Choi IW, Kim YS, Kim DK, Choi JH, Seo KH, Im SY, Kwon KS, Lee MS, Ha TY, Lee HK. Platelet-activating factor-mediated NF-kappaB dependency of a late anaphylactic reaction. J Exp Med. 2003;198:145-51.

▌ **Kobi Sade**

The Allergy & Asthma Center
Tel-Aviv Sourasky Medical Center
6 Weizman Street
Tel-Aviv, 64239 Israel
E-mail: kobisade@gmail.com