Spleen Damage in Endotoxaemic Mice: The Involvement of Nitric Oxide

The association between *Escherichia coli* endotoxin-induced organ damage and nitric oxide-related mechanisms was investigated in the spleen of male Swiss albino mice (20—40 g) by using (1) Pt/Ir electrochemical sensor connected to an amperometric detection system (NO-501, InterMedical Co., Japan), (2) nitrotyrosine immunohistochemistry, (3) conventional light microscopy and (4) immunoblotting techniques in parallel. 1 h before endotoxin injection, animals were pretreated with either nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME, 20 mg kg⁻¹, i.p.) or inducible nitric oxide synthase expression inhibitor, dexamethasone (5 mg kg⁻¹, i.p.) or the inhibitor of murine inducible nitric oxide synthase in vivo, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, 1 mg kg⁻¹, i.p.). 5 h after endotoxin treatment, electrochemically detected concentration of nitric oxide was significantly elevated (nM, endotoxin: 716.6 ± 178.2, n = 10 vs saline: 209.4 ± 127.8, n = 9, P = 0.0312, unpaired Student’s t-test) and remained so throughout the 30 min monitorization period. Neither dexamethasone nor AMT blocked the endotoxin-induced overproduction of nitric oxide indicating that the enhanced inducible nitric oxide synthase activity cannot be the only explanation. When dexamethasone and L-NAME combination was used to block both the constitutive and the inducible isoforms, nitric oxide production was virtually abolished, indicating a significant contribution from the constitutive isoform of nitric oxide synthase. The results of nitrotyrosine immunohistochemistry and the conventional light microscopy were also in agreement with the amperometric method while immunoblotting revealed the expression of both the endothelial and the inducible isoforms of nitric oxide synthase were induced in endotoxaemic animals. Thus, we conclude that endotoxin-induced splenic damage in endotoxaemia can be explained by enhanced production of nitric oxide due to the induction of both endothelial and inducible nitric oxide synthases while causal relationship and the roles of other deleterious mediators such as oxygen-derived free radicals are yet to be established.

**Key words:** Septic shock, Electrochemical sensor, Amperometry, Immunoblotting, Nitrotyrosine immunohistochemistry, Nitric oxide synthase blockade
INTRODUCTION

Despite the key involvement of nitric oxide in the hypotension and vascular hyporesponsiveness which are the hallmarks of septic shock (for review, see 1), its decisive role in the accompanying multiple organ dysfunction is still unclear (2, 3). Since endotoxin is the major stimulant of the inducible isoform of nitric oxide synthase (iNOS) which produces copious amounts of nitric oxide (4) to inflict extensive tissue damage (5), it is likely to obtain some benefit by iNOS inhibition in sepsis-related pathologies (6). Although there exists a considerable amount of information supporting this view (for review, see 7), the fact that iNOS-knockout mice suffered as much endotoxin-induced liver damage as normal mice (8) and the failure of nitric oxide donors to mimic fully the effects of endotoxin (9), oppose this assumption. Additionally, the ineffectiveness of a relatively selective inhibitor of iNOS, L-canavanine, in preventing endotoxin-induced spleen damage (10) or mortality (11) mandates the need for adopting a concerted methodology for better understanding the role of nitric oxide in endotoxin-induced organ damage.

By taking into consideration that (i) nitric oxide is a highly reactive molecule which rapidly interacts with various molecules (e.g., oxygen-derived free radicals) and (ii) the limitations of the techniques currently available to us in accurately measuring the true amount of nitric oxide generated in the tissues (12), we decided to investigate the contribution of nitric oxide-related mechanisms in endotoxin-induced tissue damage by using direct and indirect techniques concurrently.

Thus, we opted for the spleen as the target organ in endotoxemic mice due to our previous expertise (10) and its ease of access. With the help of a commercially available amperometric nitric oxide measuring system that uses a Pt/Ir sensor (Nitric Oxide Meter NO-501, Inter Medical Co., Nagoya, Japan), we attempted to monitor the nitric oxide production in the splenic pulp quantitatively in conjunction with the nitrotyrosine immunohistochemistry which reveals the presence of 3-nitro-L-tyrosine, a stable product generated by the prompt reaction of nitric oxide with the tyrosine residues of the proteins (13). The comprehensive approach in the present study was further augmented by the incorporation of immunoblotting assessment performed to reveal endothelial and inducible isoforms of nitric oxide synthase in tissue samples, together with the conventional hematoxylin and eosin histopathological examination utilized to evaluate the extent of tissue damage inflicted by endotoxin.

On the other hand, it should be emphasized that despite some encouraging reports (14, 15, 16, 17), the lack of an established confidence in the measurement of nitric oxide amperometrically (18) obliges the employment of combinatory techniques in order to increase the consistency of the outcome.
Especially, the accumulating controversial evidence from studies which indicate that (1) nitric oxide can be formed independent from the enzymes (19), (2) endothelial nitric oxide synthase can masquerade as iNOS (20), (3) endotoxin can induce endothelial isoform of nitric oxide synthase (21) within 4 h (22) contrary to what was reported previously (23) and (iv) finally, the possibility of the occurrence of tyrosine nitration in mice which lack iNOS (24) further complicates the planning of the experiments designed to correlate the amount of nitric oxide with the extent of tissue damage seen in endotoxaemia.

Therefore, we attempted to obtain a comprehensive view about the correlation of endotoxin-induced damage and the nitric oxide-related mechanisms in the spleen of endotoxaemic mice, which were also treated with various nitric oxide synthase inhibitors. For this purpose, 1 h before the injection of endotoxin, we pretreated the animals either with L-N^G-nitroarginine methyl ester (L-NAME), to inhibit the constitutive isoform of nitric oxide synthase (25) or dexamethasone, an inhibitor of the expression of the inducible nitric oxide synthase (26) or 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), an inhibitor of iNOS which is 10—40 fold more selective for murine type (27) that can be used in vivo (28). In some experiments, L-NAME and dexamethasone were used in combination to inhibit both the endothelial and inducible isoforms of nitric oxide synthase and the amount of the nitric oxide generated in the splenic pulp was then determined by using the electrochemical amperometric detection method, nitrotyrosine immunohistochemistry, conventional light microscopy and immunoblotting techniques, all in parallel.

A preliminary account of some of the initial histopathological examinations of this study were presented orally to the 13th Congress of The Polish Pharmacological Society which was held between 13—16 September 1998 in Katowice, Poland (29) and an overview of some of the amperometric measurement data were discussed at the 7th Jagiellonian Medical Research Centre Symposium that took place between 8—9 July 1999 in Cracow, Poland (30).

MATERIALS AND METHODS

Animals

Male Swiss albino mice (20—40 g) were obtained from the Laboratory Animal Husbandry Facility of Department of Pharmacology, Hacettepe University Faculty of Medicine. Animals were kept at 22 ± 3°C and 30—70% relative humidity with 12 h dark / 12 h light cycle (lights were on between 7.00 am — 7.00 pm) with access ad libitum to tap water (drinking bottle) and standard pellet dairy chow (Korkutelim Yem Sanayii, Antalya, Turkey). All experimental procedures contained in this manuscript were approved by
Hacettepe University Animal Ethics Committee prior to the start of the experiments (Decree No: 98/9). Thus, the guiding principles of The Council Of Europe Convention For The Protection Of Vertebrate Animals Used For Experimental And Other Scientific Purposes (1 July 1992) were strictly adhered to during the execution of all procedures described in this manuscript.

**General procedures**

Endotoxin derived from *Escherichia coli* (O55:B5; 10 mg kg\(^{-1}\)) or an equivalent volume (1 ml kg\(^{-1}\)) of non-pyrogenic sterile saline (NaCl 0.9%, w/v, dissolved in pyrogen-free distilled water) was injected intraperitoneally (i.p.) to animals. In some experiments, 1 h before endotoxin injection or amperometric nitric oxide measurement or tissue fixation for immunohistochemical studies, L-NAME (20 mg kg\(^{-1}\), i.p.) or dexamethasone (5 mg kg\(^{-1}\), i.p.) or AMT (1 mg kg\(^{-1}\), i.p.) or their solvents (saline, 1 ml kg\(^{-1}\), i.p.) were given either alone or in appropriate combinations. The doses and the concentrations of the drugs were chosen on the basis of the available information in the scientific literature and our previous experiences (10, 11, 30). In particular, the dose of AMT was selected on the basis of a previous study which reported an ED\(_{50}\) value of 0.2 mg kg\(^{-1}\) for inhibiting LPS induced-increase in plasma nitrite and nitrate concentrations in vivo (28).

On the other hand, monitorization of nitric oxide concentrations and tissue fixation for immunohistochemical assesment were planned to take place approximately 5 h after endotoxin administration based on the fact that iNOS activity has reached its maximum within 2—6 h after endotoxin administration (4, 32). Thus, 4 h after saline or endotoxin injection, animals were put under terminal anaesthesia by using sodium thiopental (65 mg kg\(^{-1}\), i.p.) and placed on a cork-sheet-covered operation table which resides in a Faraday cage. The presence and the permanence of a satisfactory surgical anaesthesia was assured frequently by monitoring the attenuation of responsiveness to a painful stimulus (i.e. pinching of the dorsum of the feet with a tweezer) while the body temperature (37°C) was stabilized within 0.1°C limits by using a rectal thermistor probe-controlled incandescent lamp (100 W) placed 30 cm above the operation table during an equilibration period of 30 min. At the end of the experiments, all animals were killed by exsanguination via the carotid artery and cervical dislocation was performed before the disposal of the carcases.

**Amperometric nitric oxide measurement**

Nitric oxide measurement was performed by using a commercially available nitric oxide meter system (NO-501, InterMedical Co., Nagoya, Japan). The
technical details about the detection principles of this device have been described previously (33). Briefly, the operation of NO-501 is based on the measurement of the redox current between the working electrode and the counter electrode. The working electrode (NOE-47, InterMedical Co., Nagoya, Japan) is made of Platinium/Iridium alloy (Pt 90% + Ir 10%, 200 mm diameter) and coated with KCl, a nitric oxide-selective resin (2 mm sensing length) and a polyurethane-silicon membrane (43 mm length). This electrode was inserted in into the splenic pulp following the left flank incision that revealed the spleen. On the other hand, the counter electrode (NOR-20, InterMedical Co., Nagoya, Japan) which is made of carbon fibers in a thin brush shape (11 mm length) was laid out on the surface of the organ in close proximity to the working electrode. Applying 0.4—0.8 V to the working electrode results in the electrochemical oxidation of nitric oxide which produces a current of picoamper magnitude that is detected, amplified and displayed by NO-501. The alterations in the current during the experiments were also recorded by using Harvard Oscillograph Pen-Recorder (Massachusetts, USA).

The calibration of the system was performed before every experiment by using S-nitroso-N-acetyl-dl-penicillamin (SNAP) as the standard nitric oxide generator according to the instructions of the manufacturer (InterMedical Co., Nagoya, Japan). Both electrodes were immersed into an organ bath maintained at 37.0 ± 0.1°C and SNAP was dissolved in phosphate buffered saline (NaCl 8.0 g, KCl 0.2 g, Na₂HPO₄ 0.7 g, KH₂PO₄ 0.2 g all dissolved in 1 L of distilled water, pH = 7.4) to obtain 10 μM, 50 μM, 100 μM, 500 μM and 1 mM dilutions which was reported to generate 13, 65, 130, 650, 1300 nM of nitric oxide (14), respectively. For each electrode, a nitric oxide concentration-current calibration curve was constructed and the corresponding tissue concentrations of nitric oxide were calculated by intrapolation within the curve.

**Hematoxylin and eosine staining**

At the end of some experiments, spleens were isolated and placed into 10 % formaldehyde solutions for 72 h. When the tissues are fixed satisfactorily, serial sections (5—6 mm thickness) were prepared from paraffin blocks and stained with hematoxylin and eosine. Histopathological examination of the slides were performed by using conventional light microscopy (Zeiss Axioskop, Germany) after appropriate blinding.

**Immunohistochemistry**

In another set of experiments, mice were allocated into appropriate groups and at the end of the drug-treatment protocols, the animals were killed by using
an overdose of sodium thiopenthal (approximately 150 mg kg⁻¹, i.p.) and perfused transcardially with 4% paraformaldehyde solution. Spleens were then removed and kept in paraformaldehyde solution (4%) for 48 h and were subsequently embedded in paraffin blocks. After 4 μm-thick slices were obtained, they were deparaffinized at 56°C overnight in xylene solution which were then hydrated in graded alcohol solutions. Sections were stained with antityrosine antibody (1/100 monoclonal, Upstate Biotechnology Inc., New York, USA) by avidin-biotin method (13). To establish the specificity of antibody binding, one set of the tissue sections were incubated with antibody mixed with 15 mmol L⁻¹ nitrotyrosine solution (dissolved in phosphate buffered saline, pH = 7.6) for 6 h at room temperature. Diaminobenzidine was used as the chromogen and hematoxylin as the counter stain for antibody. The final slices of the spleen were evaluated by an observer who is appropriately blinded.

**Immunoblotting**

In another set of experiments, the spleens were homogenised in ice-cold homogenisation buffer (50 mM Tris-HCl, pH = 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg ml⁻¹ aprotinin, 2 mM dithiothreithol, 20 mM detergent of CHAPS) by using a polytron homogeniser. Nuclei and cell debris were separated by centrifugation at 500 g for 10 min at 4°C. Supernatants were used for protein measurements. Protein quantity was measured by the method of Bradford (34). The proteins were subjected to 10% SDS-PAGE (35) and then transferred electrophoretically to nitrocellulose membrane. Immunoblotting was performed by using anti-iNOS antibody (NOS2, N-20: sc-651, Santa Cruz, USA), anti-eNOS antibody (1185—1205) (RBI, USA) or anti-eNOS antibody (NOS3, H159) (Santa Cruz, USA) (dilutions 1/1000) and enhanced chemiluminescence as described previously. Briefly, nitrocellulose membranes were incubated overnight at 4°C in phosphate buffered saline containing 3% bovine serum albumin and 8% nonfat dry milk. Blots were washed several times with the phosphate buffered saline, then incubated with antiserum at room temperature for 1—2 hr with shaking. Blots were then washed several times with phosphate buffered saline, incubated with horseradish peroxidase-labelled donkey anti-rabbit IgG (Amersham, United Kingdom) for 1 hr at room temperature. Blots were washed several times with phosphate buffered saline and incubated with enhanced chemiluminescence western blotting reagent (Amersham, United Kingdom) for 1 min and exposed to X-ray film for 15—45 s which was then developed and image-analysed with computer accordingly.
Drugs and chemicals

All drugs were prepared daily, dissolved in non-pyrogenic sterile saline and warmed to body temperature (approximately 37°C) before injection. Drug solutions were kept in dark containers until injection in order to protect them from light-induced decomposition.

The following drugs and chemicals were used: lipopolysaccharide (Escherichia coli endotoxin, Serotype O55:B5), L-N^6^-nitroarginine methyl ester (L-NAME), diaminobenzidine, eosine, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), phenyl-methylsulfonyl fluoride, apotinin, DL-dithiothreithol, (3-((3-cholamido-propyl)dimethylammonio)-1-propane-sulfonate (CHAPS), lauryl sulphate sodium salt (SDS), polyacrylamide absorbent gel (Sigma, USA), dexamethasone (Hoechst, Germany), S-Nitroso-N-acetylpenicillamine (SNAP, RBI, USA), 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, Tocris, UK), thiopental sodium (I. E. Ulagay Ilaç Sanayii, Turkey), hematoxylin, sodium chloride (Merck, USA), paraffin (Shandon, United Kingdom), formaldehyde (Carlo Erba, Italy).

Statistical analysis

The data are expressed as arithmetic means ± standard error of the mean (S.E.M.) of the number (n) of experiments. Two-way analysis of variance (ANOVA) for repeated measurements was used to analyse the differences between the nitric oxide concentration-time curves obtained from various experimental groups. When P < 0.05, the differences were considered to be statistically significant. To assess the calibration procedures, regression analysis were performed to evaluate the linearity of electrical current-nitric oxide concentration relation for each electrode before the experiment. Electrodes that lack linearity in their current responses to serial nitric oxide concentrations were excluded from the study.

RESULTS

Endotoxin-induced nitric oxide production in amperometric measurements

Amperometric measurement of nitric oxide in the splenic pulp indicated a basal nitric oxide production of approximately 200 nM in saline-treated control animals (n = 9) while endotoxin-treated animals (n = 10) expressed significantly (P = 0.03101, two-way ANOVA for repeated measurements)
increased levels of nitric oxide that remained quite stable throughout the monitorization period of 30 min (Fig. 1).

**Effect of nitric oxide synthase inhibition in amperometric measurements**

When the animals were treated with dexamethasone (5 mg kg$^{-1}$, i.p.) 1 h before endotoxin administration to inhibit the endotoxin-induced expression of the inducible nitric oxide synthase, the amount of nitric oxide production remained elevated as recorded by the amperometric method (Fig. 1). Although dexamethasone appeared to decrease the production of nitric oxide in saline-treated control animals, the difference between the curves did not reach

![Graph](image_url)

*Fig. 1. The concentration of nitric oxide (nM, calculated by interpolation) measured amperometrically for 30 min in the spleen of male Swiss albino mice, 5 h after saline (SAL, 1 ml kg$^{-1}$, i.p., open symbols) or endotoxin (ETX, 10 mg kg$^{-1}$, i.p., solid symbols) administration which were also treated with L-NAME (20 mg kg$^{-1}$, i.p.) or dexamethasone (DEX, 5 mg kg$^{-1}$, i.p.) 1 h before saline or endotoxin administration. Each data point indicates the arithmetic mean and the vertical bars indicate the standard error of mean of 5—10 observations at that time point. Two-way ANOVA for repeated measurements analysis applied to saline vs endotoxin curves indicated a significant difference ($P = 0.03101$) between them while no significant difference were observed between endotoxin vs endotoxin + dexamethasone ($P =$ approximately 1), saline vs saline + dexamethasone ($P = 0.60635$) or saline vs endotoxin + dexamethasone + L-NAME ($P = 0.5535$) curves.*
However, when both the constitutive and the inducible forms of nitric oxide synthase enzymes were inhibited by administering L-NAME (20 mg kg\(^{-1}\), i.p.) in addition to dexamethasone (5 mg kg\(^{-1}\), i.p.), it was possible to lower the nitric oxide production to the levels obtained from saline-treated control animals in amperometric monitorization experiments (Fig. 1).

On the other hand, AMT (1 mg kg\(^{-1}\), i.p.), an inducible nitric oxide synthase inhibitor with 10—40 fold more selectivity towards the murine type, also failed to block the effect of endotoxin in amperometric measurements (Fig. 2). In fact, AMT itself appeared to increase the production of nitric oxide based on the observation that there was a significant (P = 0.00577, two-way ANOVA for repeated measurements) difference between the curves obtained from endotoxin vs endotoxin + AMT treated animals.

![Fig. 2. The concentration of nitric oxide (nM, calculated by interpolation) measured amperometrically for 30 min in the spleen of male Swiss albino mice, 5 h after saline (SAL, 1 ml kg\(^{-1}\), i.p., open symbols) or endotoxin (ETX, 10 mg kg\(^{-1}\), i.p., solid symbols) administration which were also treated with 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, 1 mg kg\(^{-1}\), i.p., broken lines) 1 h before saline or endotoxin administration. Each data point indicates the arithmetic mean and the vertical bars indicate the standard error of mean of 5—10 observations at that time point. Two-way ANOVA for repeated measurements applied to saline vs saline + AMT curves indicated a significant (P = 0.00577) difference between them while there was no significant difference (P = 0.5628) between the curves obtained from endotoxin vs endotoxin + AMT treated animals.](image-url)
saline-treated controls and saline plus AMT-treated animals (Fig. 2). However, this assumption was not entirely valid in the case of endotoxin-treated animals since the apparent elevation of nitric oxide production that can be attributed to the presence of AMT, did not reach statistical significance due to the magnitude of standard errors of the arithmetic means (P = 0.5628, two-way ANOVA for repeated measurements) (Fig. 2).

Histopathological examination

When the sections of spleen from endotoxin-treated animals were examined by using conventional light microscopy after hematoxylin and eosine staining (Figs. 3a and 3b), a profound haemocongestion was observed in the red pulp which seemingly constricted the central splenic venules in the white pulp (Fig. 3a) to disappearance (Fig. 3b) while dexamethasone treatment did not significantly modify the effects of endotoxin. On the other hand, the preparations obtained from AMT-treated animals, regardless of endotoxin co-treatment,
exhibited a variety of pathological alterations ranging from mild haemocongestion to severe intracapsular haemorrhage with intense lymphoid hyperplasia in the white pulp as observed in endotoxin treated animals. Moreover, there was a remarkable variation between the extent of tissue damage in tissue samples obtained from the same treatment groups which further complicated the categorization of the histopathological findings into a fixed template for that treatment protocol. However, the combination of L-NAME and dexamethasone treatment remarkably attenuated the effects of endotoxin (data not shown).

**Immunohistochemical examination**

The examination of splenic tissue by using paranitrotyrosine immunohistochemistry revealed focal staining in saline-treated controls (Fig. 3c) which was drastically increased both in the red and the white pulp regions of splenic slices obtained from endotoxin-treated animals (Fig. 3d). Similar to the results obtained with hematoxylin and eosine, dexamethasone treatment did not significantly modify the effects of endotoxin while L-NAME + dexamethasone combination effectively attenuated them as arbitrated by the decrease in the

![Fig. 4. The result of the immunoblotting performed to visualize the 140 kD — endothelial isoform of nitric oxide synthase (eNOS, upper panel) and the 109 kD-inducible isoform of nitric oxide synthase (iNOS, lower panel) in splenic tissue homogenates obtained from saline (1 ml kg⁻¹, i.p., Control), endotoxin (10 mg kg⁻¹, i.p., LPS), saline + dexamethasone (5 mg kg⁻¹, i.p., DEX) or endotoxin + dexamethasone (LPS + DEX) treated animals. Dexamethasone was administered 1 h before saline or endotoxin injection.](image_url)
intensity of brownish staining. In contrast, the intensity and the dissemination of staining were intensified in preparations obtained from AMT-treated animals regardless of the endotoxin co-treatment (data not shown).

**Immunoblotting studies**

*Figure 4* shows the results of immunoblotting studies performed to visually compare the amount of inducible nitric oxide synthase protein in the splenic tissue homogenates obtained from different treatment groups.

Endotoxin treatment resulted in the appearance of 140 kD-constitutive nitric oxide synthase isoform (upper panel, second column) which was not visible in the controls (upper panel, first column). In contrast, dexamethasone failed to block the effect of endotoxin (upper panel, fourth column) without having any effect in saline-treated controls (upper panel, third column) (*Fig. 4*).

Interestingly, endotoxin treatment resulted also in the appearance of 109 kD-inducible nitric oxide synthase enzyme (lower panel, second column) which was absent in the controls (lower panel, first column) while dexamethasone has successfully blocked the effect of endotoxin (lower panel, fourth column) without having any effect in saline-treated controls (lower panel, third column) (*Fig. 4*).

**DISCUSSION**

These results indicate that endotoxin stimulates the overproduction of nitric oxide in the splenic pulp by inducing the expression of both 109 kD-inducible and 140 kD-endothelial isoforms of nitric oxide synthase and it is possible to associate the extent of tissue damage to nitric oxide generation by compiling the outcome of conventional light microscopic examination with the results of electrochemical and immunohistochemical detection methods. Also, the present data emphasize the need for a concerted approach in quantitative nitric oxide determination studies performed in whole animal models of septic shock due to the intricate nature of underlying mechanisms that do not entirely conform to widely agreed patterns.

In general, our results are in agreement with the fact that nitric oxide is generated in copious amounts in endotoxaemic states (1, 2, 3, 6, 7, 32) and demonstrate for the first time that spleen can serve as a convenient organ to monitor in vivo nitric oxide production in anaesthetized animals. However, rather unfavourable signal/noise ratio of the amperometric methodology in comparison to conventional pharmacological experimental methods in general, was reflected as disturbingly magnified error bars in our figures and complicated the interpretation of the data. On the other hand, successful in vitro monitorization of 5-hydroxtryptamine-induced nitric oxide generation in
porcine coronary artery in a previous study (18) which utilized a rather different type of electrode than ours, denote the encouraging nature of such approach. Moreover, the results of the same study indicate that the signal-to-noise ratio of the Pt/Ir based amperometric electrochemical nitric oxide detection system we have used, is apparently better than that of the voltametric detection system using nickel-porphyrin based nafion-coated carbon fiber electrodes (18) although this advantage does not appear to rule out the absolute necessity for a combined methodological approach as adopted in the present study.

The importance of this attitude becomes amplified while interpreting the outcome of experiments with some drugs such as AMT, since the data obtained from these experiments do not fit in entirely to the general format, which *a priori* assumes that AMT blocks iNOS (27) to flawlessly decrease, if not abolish, the production of nitric oxide in endotoxaemic animals (28). In fact, in the present study, AMT itself has increased the amount of nitric oxide, an effect which was just the opposite of what was expected. Although we do not know the exact mechanism(s) underlying this phenomenon, previous studies which reported that nitric oxide can be generated from amino moieties via enzyme-independent mechanisms (19) depending on the redox potential of the milieu (36), and nitric oxide (37), as well as nitric oxide synthase inhibitors, can spontaneously transform into various forms having diverse biological properties (38), it can be speculated that AMT, an amino-containing drug, can also undergo such a conversion. However, this speculation must be treated cautiously until such is proven undoubtedly.

Another novel finding of this study is the results of immunoblotting experiments which revealed the induction of 140 kD-endothelial isoform of nitric oxide synthase by endotoxin. Contrary to the widely accepted original concept of endotoxin activating only iNOS (23, 39), our present findings are fully supported by recent studies indicating that endotoxin is perfectly capable of inducing the endothelial nitric oxide synthase activity within 4 h after endotoxin injection in myocardium (22), in rat brain astrocytes (21) and in bovine coronary venular endothelial cells via post-translational modification mediated through a protein tyrosine kinase cascade (40). Moreover, recent studies which indicate that transgenic mice overexpressing the endothelial isoform are resistant to the deleterious effects of endotoxin (41) and also nitric oxide generated by the endothelial isoform can masquerade as the inducible isoform in producing venodilation during endotoxaemia in human beings (20), strongly recommend that our current understanding of the interaction between endotoxin and nitric oxide synthases needs an extensive revision. Although a causal relationship between the endotoxin-induced tissue damage and the amount of nitric oxide generation remains to be established, the present data suggest that both the endothelial and inducible isoforms of nitric oxide synthase are involved.
Moreover, the results of the nitrotyrosine immunostaining experiments which were authenticated in different clinical entities (13, 42, 43) also support the outcome of other techniques utilized in this study.

In conclusion, we suggest that spleen can be used as a convenient organ to investigate the association between the deleterious effects of endotoxin and nitric oxide-related mechanisms but it is essential that a concerted methodological approach is adopted to substantiate the findings of different experimental techniques.

Acknowledgements: This study was supported by a grant from the Hacettepe University Research Fund Project Number: 97.01.101.005 and partially by NOVARTIS Farmakoloji Dali Proje Destekleri, Istanbul, Turkey. We gratefully acknowledge the technical advisory support provided by Prof. Tadeusz Malinski, PhD from Center for Biomedical Research, Oakland University, Rochester, Michigan, USA and Prof. Dr. Yun Wang, MD PhD from Department of Pharmacology, National Defense Medical Centre, Taipei, Taiwan. Also, we are grateful for the help provided by Aysun Kara-Dincel, BSc-Chemical Engineering, MSc-Biochemistry during the calibration of the nitric oxide meter NO-501.

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Received: May 5, 2001
Accepted: October 18, 2001

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