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NITRIC OXIDE DECREASES EICOSANOID PRODUCTION VIA ACTIVATION OF GUANYLYL CYCLASE AND POLYADP-RIBOSE SYNTHASE IN ENDOTOXIN-STIMULATED WHOLE BLOOD, KIDNEY AND HEART

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Abstract

Exposure to endotoxin in vivo or in vitro activates nitric oxide (NO) by inducible NO synthase (iNOS) as well as guanylyl cyclase (GC), cyclo-oxygenase (COX) and polyADP-ribose synthase (PARS) in several cell types. The aim of this study was to invesigate whether iNOS-derived NO could interact with COX via activation of GC or PARS enzymes in whole blood, kidney and heart culture. Endotoxin caused increased levels of nitrite without any effect on lipid peroxidation in whole blood and tissues. The endotoxin-induced increase in nitrite production was decreased by aminoguanidine (iNOS inhibitor), methylene blue (GC inhibitor), indomethacin (COX inhibitor) or 3-aminobenzamide (PARS inhibitor) in blood and tissues while certain concentrations of the agents, except for indomethacin, also increased nitrite production in blood. Endotoxin caused a decrease in thromoboxane B2 (TxB2) production in blood and kidney. Aminoguanidine reversed the effect of endotoxin in blood. The endotoxin-induced levels of TxB2 were further decreased by indomethacin in kidney. These results suggest that blockade of GC, COX or PARS pathways decreases, increases or does not change nitrite production depending on the degree of inhibition and tissue type. iNOS-derived NO may decrease eicosanoid production via activation of GC or PARS enzymes.

Key Words: *Endotoxin, nitric oxide, mice, blood, kidney, heart*

Endotoksin Ile Uyarılan Tam Kan, Böbrek Ve Kalpte Nitrik Oksit Guanilil Siklaz ve Poli-ADP-Riboz Sentazı Aktive Ederek Eikozanoit Oluşumunu Azaltmaktadır

Endotoksine in vivo veya in vitro maruziyet çeşitli hücre tiplerinde indüklenebilir nitrik oksit (NO) sentaz (iNOS), guanilil siklaz (GS), siklo-oksijenaz (COX) ve poli-ADP-riboz sentaz (PARS)'yi aktive etmektedir. Bu çalışmanın amacı, tam kan, böbrek ve kalp kültüründe, iNOS kaynaklı NO'nun GS veya PARS enzimlerini aktive ederek COX ile etkileşip etkileşmediğini araştırmaktadır. Endotoksin, tam kan ve dokularda lipit peroksidasyonu üzerinde bir etki oluşturmaksızın, nitrit düzeylerinde artmaya neden olmuştur. Endotoksin ile kan ve dokularda artan nitrit oluşumu aminoguanidin (iNOS inhibitörü), metilen mavisi (GS inhibitörü), indometazin (COX inhibitörü) veya 3-aminobenzamit (PARS inhibitörü) ile azalırken, indometazin dışında, bu maddeler bazı konsantrasyonlarında kanda nitrit oluşumunu artırmıştır. Endotoksin kan ve böbrekte tromboksan B_2 (TxB_2) oluşumunda bir azalmaya neden olmuştur. Endotoksinin kandaki bu etkisini aminoguanidin geri çevirmiştir. Endotoksin ile böbrekte oluşan TxB_2 düzeyleri indometazin ile daha da azalmıştr. Bu bulgular, GS, COX veya PARS yollarının blokajının inhibisyonun derecesi ve doku tipine bağlı olarak nitrit oluşumunu azalttığı, artırdığını veya değiştirmediğini düşündürmektedir. iNOS kaynaklı NO, GS veya PARS enzimlerini aktive ederek eikozanoit oluşumunu azaltabilir.

Anahtar Kelimeler: *Endotoksin, nitrik oksit, fare, kan, böbrek, kalp*

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INTRODUCTION

Nitric oxide (NO) is produced enzymatically in numerous tissues and considered as important mediator of many physiological and pathophysiological processes (1). NO is synthetised from L-arginine by three isoforms of the enzyme NO synthase (NOS) (2). Of the three established isozymes of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutive (cNOS), low-output, calcium-activated enzymes whose physiological function is signal transduction. The third form of NOS, inducible NOS (iNOS), is constitutively expressed only in the selected tissues such as lung epithelium, and more typically it is synthetised in response to inflammatory or pro-inflammatory mediators. iNOS is mainly regulated at the level of transcription, but post-transcriptional and translational mechanisms that regulate protein expression of this isoform have been described. The stimuli and conditions that determine iNOS expression are cell- and species-specific (1-3).

Excessive production of NO following the induction of iNOS as a result of an increased circulating cytokine concentration makes a major contribution to the development of the characteristic symptoms of inflammatory conditions such as endotoxic shock (3). However, it is evident that in many cases an increase in NO by itself may be insufficient, and the generation of other mediators, such as reactive oxygen-centred radicals, may also play a crucial role in the tissue dysfunction and damage observed in septic shock, multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF) including kidney, liver, brain and lungs, and death of patient. Soluble guanylyl cyclase (GC) is thought to be the most important receptor for NO as a signalling molecule. Binding of NO to heme group of GC leads to enormous stimulation of cyclic guanosine monophosphate (cGMP) production, yielding an up to 400-fold activation of the purified enzyme. Similar to iNOS, co-induction of inducible cyclooxygenase (COX-2) by endotoxin and/or cytokines resulted in an elevated production of proinflammatory prostaglandins which are linked to MODS and MOF (4-7). Reactive oxygen-centered radicals, such as superoxide anion have been proposed as powerful triggers of DNA single strand breakage, and consequent activation of the cell suicide cycle by polyADP-ribose synthase (PARS), a nuclear repair enzyme in various cell types *in vitro*. PARS activation, most likely due to peroxynitrite production by the reaction between NO and superoxide anion, is also responsible for the cellular energetic derangement and ultimately cell death observed during endotoxic shock (8).

Lipid peroxidation is also one of the basic mechanisms involved in cell and tissue damage. The regulation of nonenzymatic and enzymatic lipid peroxidation reactions by NO reveals novel non-cGMP-dependent reactivities for NO. NO and its metabolites stimulate and inhibit lipid peroxidation reactions, modulate enzymatically catalyzed lipid oxidation, complex with lipid-reactive metals. Through these mechanisms, NO can regulate enzymatic lipid peroxidation and the production of inflammatory and vasoactive eicosanoids by COX and lipoxygenase (9). High reactivity of NO with radicals may be beneficial *in vivo*, such as scavenging peroxyl radical and inhibiting lipid peroxidation.

Since there are conflicting reports on the interactions between the NOS and COX pathways (10,11), we examined whether iNOS-derived NO could interact with COX via activation of GC or PARS enzymes in whole blood, kidney and heart. Effects of endotoxin and inhibitors of the enzymes on eicosanoid production and lipid peroxidation were also studied.

EXPERIMENTAL

Animals

Male Balb/c mice (Ankara University Faculty of Medicine Department of Pharmacology, Ankara, Turkey), weighing 20-40 g were used throughout the experiments according to the proposals of the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed in standard transparent cages (20 per cage) with free access to food and water under environmental controlled conditions at 24 ± 2° C. They were synchronised by maintenance of controlled environmental conditions for at least 2 weeks prior to and throughout the duration of the experiments. The circadian rhythmicity of the animals were entrained by a standardised 12 h light/dark cycle (lights on at 9:00 a.m.) with a light intensity of approximately 100 lux. Automatic timer controlled cool fluorescent bulbs were used to provide lighting.

Preparation of whole blood and organ culture

Blood was obtained from mice after cervical dislocation at 9:00-10:00 a.m. After the blood samples were collected into 1.5 ml sterile tubes containing 1000 U/ml heparine, kidney and heart were isolated and washed with phosphate-buffered saline (PBS, pH 7.4); 50 µl aliquots of blood were pipetted into sterile flat-bottom 96-well tissue culture plate wells (0.32 cm^2) pre-loaded with 250 µl Dulbecco's modified Eagle medium (DMEM) containing phenol red (15 mg/l) supplemented with D-glucose (1 g/l), L-glutamine (0.58 g/l) and streptomycin (100 g/l). To induce NOS in blood cells, endotoxin at 10, 30, 100 or 300 µg/ml of DMEM was added to the wells. Kidney and heart were also isolated from mice and washed with PBS in a petri dish. Then, the whole organs were placed into sterile tissue culture dishes (8.7 cm^2) containing 4 ml of DMEM with or without endotoxin at 1, 3 or 10 µg/ml concentrations. Blood and organ cultures were incubated at 37° C, in an atmosphere containing 5% CO_2 in a culture incubator (IG 150 CO_2 incubator, Jouan, France). At 3, 6, 9, 12, 24 and 48 h, culture medium was collected and frozen at -80° C. As an index for iNOS activity, nitrite concentrations in the medium were measured. The time having elapsed till a statistically significant difference in nitrite levels in the absence and presence of endotoxin was selected as an incubation period for blood and each organ. Endotoxin concentrations to increase levels of nitrite were also selected experimentally. Then, the cultures were incubated with aminoguanidine (selective iNOS inhibitor) (0.1, 1, 3, 10, 30, 100, 300, 1000 and 3000 µM), methylene blue (GC inhibitor) (0.001, 0.01, 0.1, 1 and 10 µM), indomethacin (nonselective COX inhibitor) (0.001, 0.01, 0.1, 1, 3, 10, 30, 100, 300 and 1000 µM) or 3-aminobenzamide (selective PARS inhibitor) (0.001, 0.01, 1, 10, 30, 100, 300 and 1000 µM) in the presence of endotoxin. At selected time points, culture medium was collected and frozen. At 48 h, viability of polymorphonuclear leucocytes in blood culture, assessed by the two-color flow cytometric assay using acridine orange and ethidium bromide, was greater than 80% in the absence and presence of endotoxin.

Nitrite measurement

In biological systems conversion of NO in aqueous solution to nitrite and nitrate is thought to favour nitrite production (12). It has been reported that nitrite is the only stable end-product of the autooxidation of NO in aqueous solution (13) and measurement of nitrite concentrations in the culture medium widely accepted as an index for NOS activity (14,15). Therefore, concentrations of nitrite in the culture medium were measured by using the diazotization method based on the Griess reaction, which is an indirect assay for NO production (16). Briefly, samples (100

µl) were pipetted into 96-well tissue culture plates and an equal volume of Griess reagent (1% sulphanylamide (50 µl) and 0.1% N-1-naphtylethylenediamine dihydrochloride (50 µl) in 2.5% ortophosphoric acid) was added to each well. After incubation for 15 min at room temperature, absorbance was measured at 550 nm with a microplate reader (Diagnostic Pasteur, LP 400, Germany). Nitrite concentration calculated by a standard calibration curve of sodium nitrite solutions in DMEM.

Thromboxane B_2 (TxB_2) measurement

As an index for COX activity, TxB_2 concentrations were measured in the homogenates by ELISA according to the manufacturer's instructions using the TxB_2 assay kit (Cayman Chemical Co., USA) with a microplate reader (ELX-800, Bio-Tek, USA).

Malondialdehyde (MDA) measurement

As an index for lipid peroxidation and free radical generation, MDA concentrations in the culture medium were measured using by thiobarbituric acid reactivity method (17). Briefly, 50 µl of sample was added to a mixture containing 200 µl PBS, 6.5 µl buthylated hydroxytoluene and 125 µl trichloroacetic acid. After mixing, samples were kept at + 4° C for 2 h and then centrifuged at 4000 rpm for 15 min. 250 µl of the supernatant was added to a mixture 19 µl EDTA-NaH_2O and 63 µl thiobarbituric acid and then boiled for 15 min. The absorbance of the mixture was measured at 490 nm with a microplate reader (ELX-800, Bio-Tek, USA). MDA concentrations in the samples were calculated by a standard calibration curve of 1,1,3,3-tetraethoxypropane prepared in the same manner.

Drugs

Endotoxin (**Escherichia coli** O111:B4 lipopolysaccharide), L-glutamine, aminoguanidine, methylene blue, 3-aminobenzamide, indomethacin, acridine orange, ethidium bromide, sodium nitrite, buthylated hydroxytoluene, EDTA-Na_2H_2O, 2-thiobarbituric acid, NaOH and 1,1,3,3-tetraethoxypropane were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other agents used were DMEM (Biochrome KG, Seromed, Berlin, Germany), streptomycin sulfate (Wyeth İlaçları A.Ş., İstanbul, Turkey), absolute alcohol (Riedel-deHaen, Seelze, Germany), and trichloroacetic acid (J.T. Baker, Deventer, The Netherlands). All other chemicals were obtained from Merck (Darmstadt, Germany). Indomethacin was dissolved and diluted in 5% $NaHCO_3$. Other agents were dissolved and diluted in distilled water. All solutions were prepared daily.

Data analysis

The results are reported as the means ± SEM. n refers to the number of animals used. Statistical comparisons were made using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California U.S.A., http://www.graphpad.com) by one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons or Kruskal-Wallis test followed by Dunn's test. Student's t or Mann-Whitney U tests were also used when necessary. P value of < 0.05 is considered statistically significant. Differences are considered significant if $p < 0.05$.

RESULTS

Effect of endotoxin on NO production

Blood and kidney, but not in heart, maintained in culture for up to 48 h released detectable amounts of nitrite throughout the time course with the highest release being detected at 48 h (**P** < 0.05) (Table 1). Addition of endotoxin to the culture medium resulted in a gradual increase in nitrite levels during 48 h (**P** < 0.05), however, the concentrations of endotoxin necessary to increase the levels of nitrite were different in blood, kidney and heart. In whole blood culture, 10, 30 or 100 μg/ml of endotoxin had no effect on the levels of nitrite during 48 h (data not shown). When endotoxin was used at 300 μg/ml concentration, the levels of nitrite were higher than control values at 48 h (**P** < 0.05). On the other hand, endotoxin was able to increase levels of nitrite at much lower concentrations in kidney and heart than in blood. The levels of nitrite induced by 10 μg/ml of endotoxin in kidney and heart was higher than control at 48 h (**P** < 0.05). On the other hand, the endotoxin-induced levels of nitrite were lower than the control group in kidney at 3 h, in heart at 6, 9 and 12 h (**P** < 0.05).

From these results, concentrations of endotoxin to increase levels of nitrite were determined as 300 μg/ml for blood and 10 μg/ml for kidney and heart. The incubation period for blood, kidney and heart was also determined as 48 h after endotoxin administration.

Table 1. Endotoxin-induced concentrations of nitrite (μM) in whole blood, kidney and heart culture medium

	Incubation time (h)					
	3	**6**	**9**	**12**	**24**	**48**
Blood						
Control	35.4±7.1 (n= 10)$^{+}$	24.8±7.6 (n= 7)$^{+}$	26.8±6.3 (n= 8)$^{+}$	26.0±7.1 (n= 7)$^{+}$	33.0±7.6 (n= 6)$^{+}$	174.1±21.7 (n= 15)
Endotoxin (300 μg/ml)						
	26.8±4.8 (n= 12)	25.0±4.9 (n= 12)	22.7±4.3 (n= 12)	22.8±4.0 (n= 12)	34.6±6.0 (n= 12)	224.3±11.5* (n= 19)
Kidney						
Control	0.06±0.01 (n= 14)$^{+}$	0.19±0.04 (n= 14)$^{+}$	0.13±0.03 (n= 14)$^{+}$	0.17±0.03 (n= 14)$^{+}$	0.53±0.08 (n= 20)$^{+}$	0.89±0.12 (n= 24)
Endotoxin (10 μg/ml)						
	0.02±0.01* (n= 14)	0.14±0.03 (n= 14)	0.07±0.03 (n= 14)	0.40±0.11 (n= 14)	0.73±0.09 (n= 19)	1.29±0.16* (n= 21)
Heart						
Control	0.21±0.03 (n= 6)	0.40±0.05 (n= 7)	0.39±0.08 (n= 7)	0.22±0.06 (n= 7)	0.83±0.02 (n= 6)	1.04±0.16 (n= 8)
Endotoxin (10 μg/ml)						
	0.25±0.02 (n= 5)	0.05±0.02* (n= 7)	0.13±0.04* (n= 7)	0.02±0.01* (n= 7)	0.85±0.14 (n= 7)	1.71±0.19* (n= 11)

All values are presented as the means ± SEM. n refers to the numbers of animals used per group. $^{+}P < 0.05$ *vs* 48 h. $^{*}P < 0.05$ *vs* control.

Effect of aminoguanidine on endotoxin-induced increase in NO production

In order to confirm the endotoxin-induced levels of nitrite derived from NO by iNOS, selective iNOS inhibitor, aminoguanidine, was added to the culture medium. In blood, only 300 µM of aminoguanidine inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 1a) while other concentrations of aminoguanidine did not affect the levels (257.3±32.4, 256.5±20.7; 243.6±29.69; 208.1±13.1 and 218.7±20.6 µM, n= 8-20, at 0.1, 1, 10, 1000 and 3000 µM, respectively). In kidney, aminoguanidine at 1 and 30 µM concentrations inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 2a). The inhibition at 30 µM was higher than at 1 µM (P values for 1 and 30 µM concentrations of aminoguanidine were 0.0315 and 0.0199, respectively). 10 µM aminoguanidine had no effect on the endotoxin-induced levels of nitrite (1.55±0.20 µM, n= 10). In heart, 1 µM of aminoguanidine inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 3a) while 3 and 10 µM concentrations did not change the levels (1.81±0.48 and 1.61±0.28 µM, n= 8). The levels induced by aminoguanidine alone at the concentrations, that inhibited or enhanced the endotoxin-induced increase in the levels of nitrite, were not different from control values (data not shown).

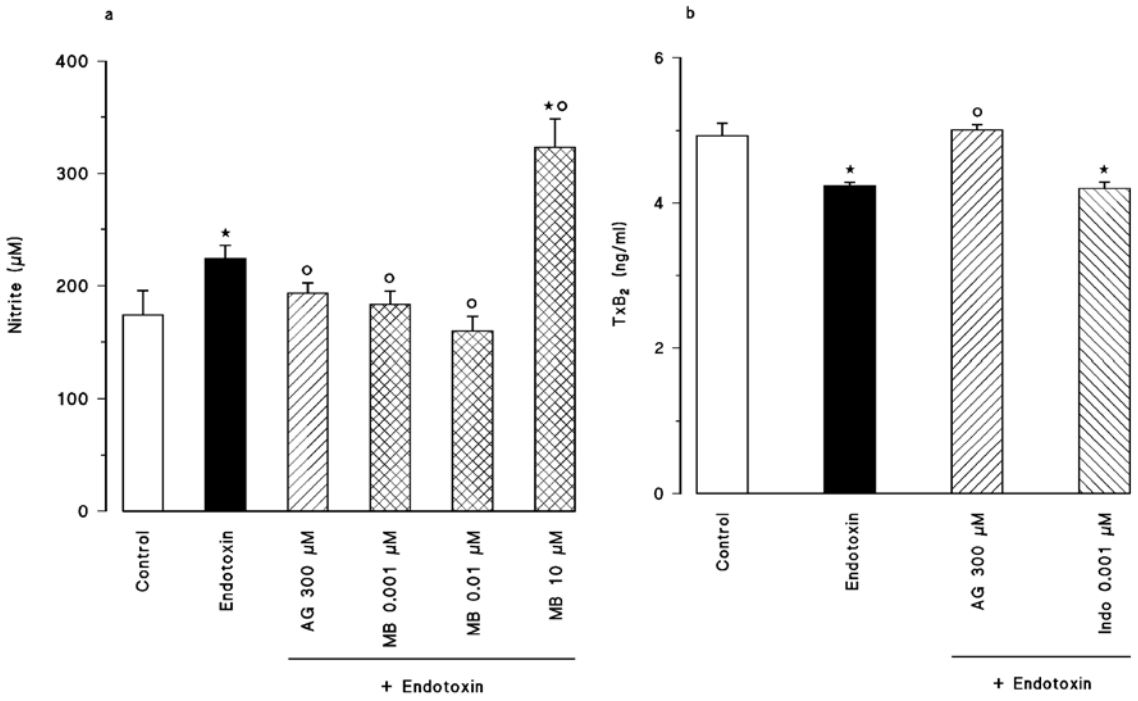

Figure 1. Effects of aminoguanidine (AG), methylene blue (MB) and indomethacin (Indo) on endotoxin (300 µg/ml)-induced levels of nitrite (a) and TxB_2 (b) in the blood culture medium during 48 h. n= 8-19. * $P < 0.05$ *vs* control. ^{o} $P < 0.05$ *vs* endotoxin.

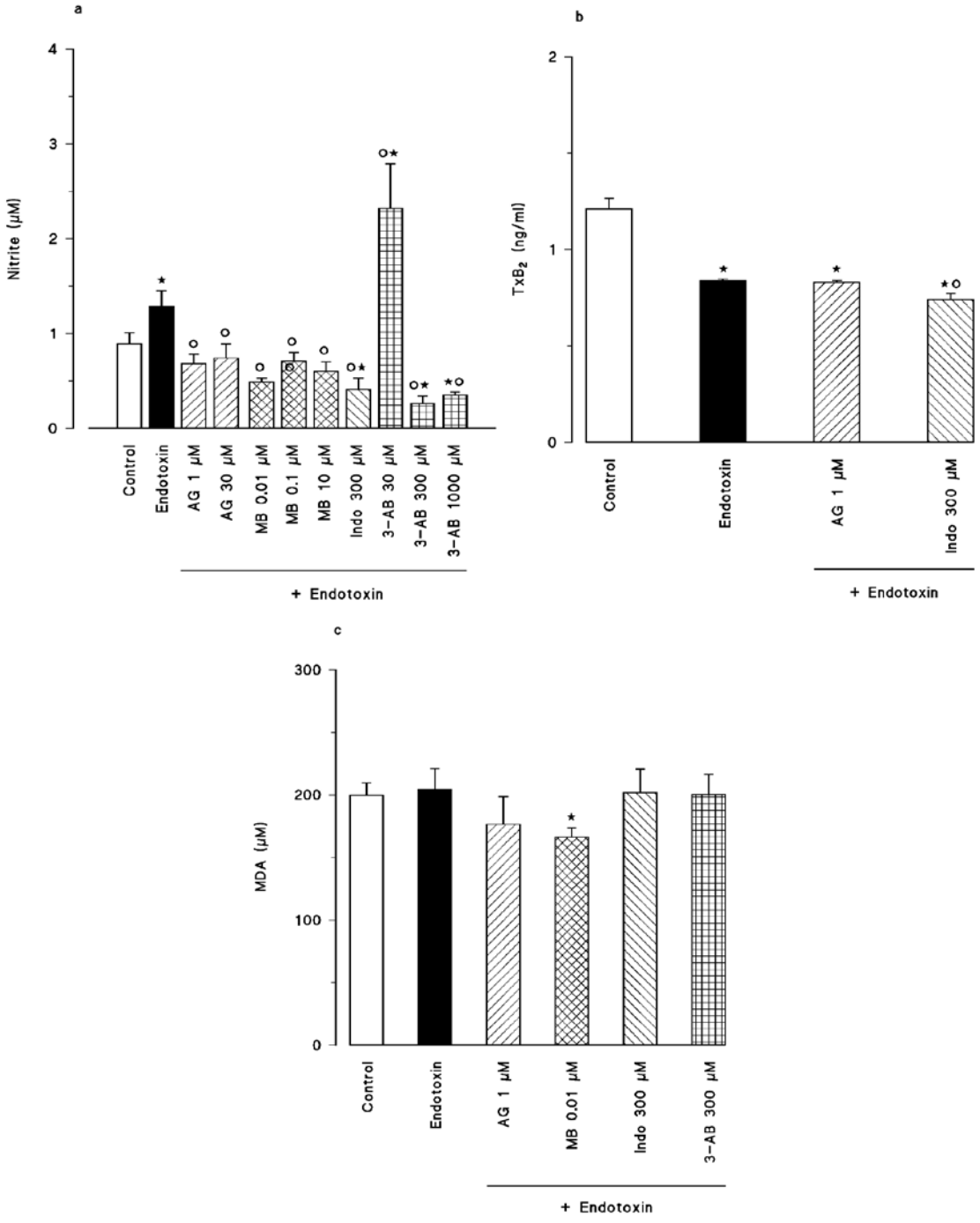

Figure 2. Effects of aminoguanidine (AG), methylene blue (MB), indomethacin (Indo) and 3-aminobenzamide (3-AB) on endotoxin (10 μg/ml)-induced levels of nitrite (a), TxB_2 (b) and MDA (c) in the kidney culture medium during 48 h. n= 3-24. * $P < 0.05$ *vs* control. º $P < 0.05$ *vs* endotoxin ($P < 0.05$).

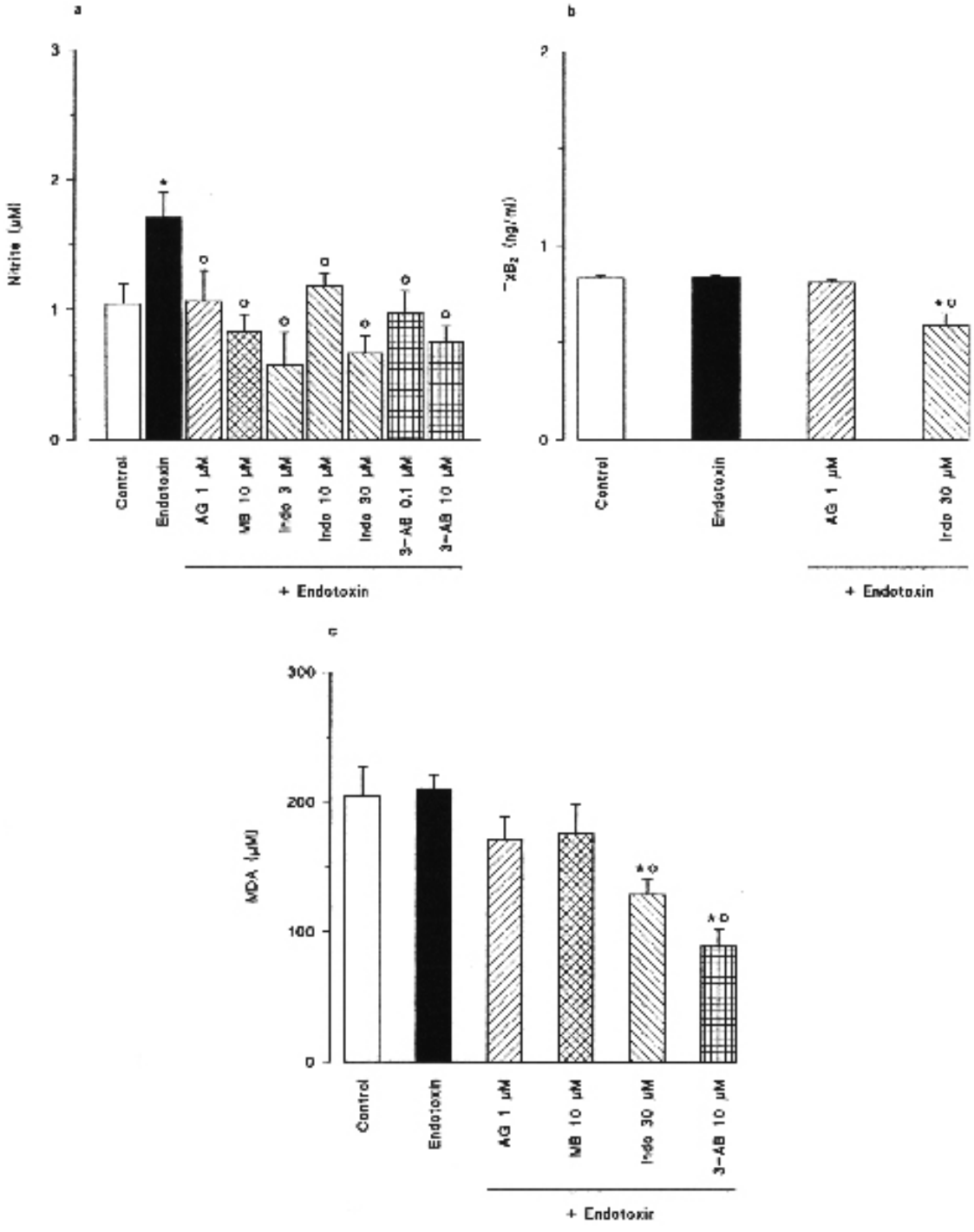

Figure 3. Effects of aminoguanidine (AG), methylene blue (MB), indomethacin (Indo) and 3-aminobenzamide (3-AB) on endotoxin (10 µg/ml)-induced levels of nitrite (a), TxB_2 (b) and MDA (c) in the heart culture medium during 48 h. n= 4-12. * $P < 0.05$ *vs* control. º $P < 0.05$ *vs* endotoxin ($P < 0.05$).

Effect of methylene blue on endotoxin-induced increase in NO production

In order to evaluate the contribution of GC to endotoxin-induced increase in the levels of nitrite, GC inhibitor, methylene blue was added to the culture medium. In blood, methylene blue at 0.001 and 0.01 μM inhibited the endotoxin-induced increase in the levels of nitrite. The inhibition at 0.01 μM was higher than at 0.001 μM (*P* values for 0.01, 0.1 and 10 μM concentrations of methylene blue were 0.0013 and 0.0283, respectively). However, methylene blue caused an increase in the endotoxin-induced levels of nitrite at 10 μM concentration ($P < 0.05$); the levels induced by methylene blue was also higher than control group ($P < 0.05$) (Figure 1a). On the other hand, at 0.1 and 1 μM concentrations, it had no effect on the endotoxin-induced increase in the levels of nitrite (220.20±18.67 and 235.30±19.36 μM, n= 8, respectively). In kidney, methylene blue at 0.01, 0.1 and 10 μM concentrations inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 2a) while at 0.001 μM concentration, had no effect on the levels (0.81±0.07 μM, n= 7). The inhibition at 0.01 μM was higher than at 0.1 and 10 μM (*P* values for 0.01, 0.1 and 10 μM concentrations of methylene blue were 0.0086, 0.0392 and 0.0162, respectively). In heart, 10 μM of methylene blue inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 3a). The levels induced by methylene blue alone at the concentrations, that inhibited or enhanced the endotoxin-induced increase in the levels of nitrite, were not different from control values (data not shown).

Effect of indomethacin on endotoxin-induced increase in NO production

In order to determine the interaction between COX and iNOS, nonselective COX inhibitor, indomethacin, was added to the culture medium. In blood, indomethacin at 0.001, 0.01, 0.1, 1, 10 and 100 μM concentrations had no effect on endotoxin-induced increase in the levels of nitrite (208.80±26.52, 212.20±14.34, 217.60±17.45, 213.50±25.60, 237.20±26.67 and 252.40±23.01 μM, n= 8-20, respectively). In kidney, indomethacin at 300 μM concentration inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$); the levels induced by indomethacin was also lower than control group. (Figure 2a). On the other hand, indomethacin at 10, 30, 100 1000 μM concentrations had no effect on the levels (1.82±0.33, 1.52±0.49, 0.96±0.30 and 0.94±0.09 μM, n= 8-10, respectively). In heart, indomethacin at 3, 10 and 30 μM concentrations inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 3a) while at 100 μM concentrations did not change the levels (1.21±0.26 μM, n= 8). The inhibition at 30 μM was higher than at 3 and 10 μM (*P* values for 3, 10 and 30 μM concentrations of indomethacin were 0.0021, 0.0412 and 0.0002, respectively). The levels induced by indomethacin alone at the concentrations, that inhibited the endotoxin-induced increase in the levels of nitrite, were not different from control values (data not shown).

Effect of 3-aminobenzamide on endotoxin-induced increase in NO production

In order to determine the effect of PARS inhibition on endotoxin-induced increase in the levels of nitrite, selective PARS inhibitor, 3-aminobenzamide, was added to the culture medium. In blood culture, 3-aminobenzamide at 0.001, 0.01, 0.1, 1 and 10 μM concentrations had no effect on the endotoxin-induced increase in the levels of nitrite (207.30±16.55, 193.90±14.19, 257.30±22.97, 267.50±28.32 and 267.50±22.15 μM, n= 8-20, respectively) (Figure 1a). In kidney, 3-aminobenzamide at 300 and 1000 μM concentrations inhibited the endotoxin-induced increase

in the levels of nitrite ($P < 0.05$); the levels induced by 3-aminobenzamide was also lower than control group (Figure 2a). The inhibition at 300 μM was higher than at 1000 μM (P values for 300 and 1000 μM concentrations of 3-aminobenzamide were 0.0006 and 0.0025, respectively). On the other hand, 3-aminobenzamide at 30 μM concentration it increased the endotoxin-induced levels of nitrite ($P < 0.05$); the levels induced by 3-aminobenzamide was also higher than control group. 3-Aminobenzamide at 10 and 100 μM concentrations did not change the endotoxin-induced levels of nitrite (2.11±0.49 and 1.24±0.51 μM, n= 8-10, respectively). In heart, 3-aminobenzamide at 0.1 and 10 μM concentrations inhibited the endotoxin-induced increase in the levels of nitrite ($P < 0.05$) (Figure 3a). The inhibition at 10 μM was higher than at 0.1 μM (P values for 0.1 and 10 μM concentrations of indomethacin were 0.0129 and 0.0008, respectively). The levels induced by 3-aminobenzamide alone at the concentrations, that inhibited or enhanced the endotoxin-induced increase in the levels of nitrite, were not different from control values (data not shown).

Effect of iNOS or GC inhibitors on endotoxin-induced TxB_2 production

In order to determine the effect of iNOS or COX inhibition on the endotoxin-induced eicosanoid production, levels of TxB_2 were measured in the culture medium in the presence of aminoguanidine or indomethacin. In blood, endotoxin caused a decrease in the levels of TxB_2 that was increased by aminoguanidine at 300 μM concentration that inhibited the endotoxin-induced increase in the levels of nitrite (**P** < 0.05). On the other hand, the endotoxin-induced decrease in the levels of TxB_2 was not changed by the lowest concentration (0.001 μM) of indomethacin (Figure 1b). In kidney, endotoxin also decreased the levels of TxB_2 that was further decreased by 300 μM of indomethacin that inhibited the endotoxin-induced increase in the levels of nitrite (**P** < 0.05) (Figure 2b). On the other hand, 1 μM of aminoguanidine, that caused more significant inhibition on endotoxin-induced increase in the levels of nitrite, was not changed the endotoxin-induced levels of TxB_2. In heart, endotoxin did not change the levels of TxB_2 (Figure 3b). The endotoxin-induced levels of TxB_2 were further reduced by indomethacin at 1 μM concentration (**P** < 0.05), that caused more significant inhibition on endotoxin-induced increase in the levels of nitrite. The levels induced by indomethacin were also lower than control group (**P** < 0.05). Aminoguanidine at 1 μM concentration, that inhibited the endotoxin-induced increase in the levels of nitrite, did not changed the endotoxin-induced levels of TxB_2. The levels induced by these agents alone at the concentrations, that inhibited the endotoxin-induced increase in nitrite levels, were not different from control values (data not shown).

Effects of iNOS, GC, COX or PARS inhibitors on endotoxin-induced MDA levels

In order to establish the effects of iNOS, GC, COX or PARS inhibition on endotoxin-induced lipid peroxidation and role of free radicals, MDA levels were measured in the culture medium in the presence of aminoguanidine, methylene blue, indomethacin or 3-aminobenzamide. Endotoxin did not increase or decrease the levels of MDA in the kidney (Figure 2c) and heart (Figure 3c). In kidney (Figure 2c), aminoguanidine and 3-aminobenzamide at 1 and 300 μM concentrations, that caused more significant inhibition on the endotoxin-induced increase in the levels of nitrite, did not changed the endotoxin-induced levels of MDA. On the other hand, the levels of MDA induced by 0.01 μM of methylene blue, that caused more significant inhibition on the endotoxin-induced increase in the levels of nitrite, was lower than control (**P** < 0.05). Indomethacin at

300 μM concentration, that inhibited the endotoxin-induced increase in the levels of nitrite, did not changed the endotoxin-induced levels of MDA. In heart (Figure 3c), aminoguanidine and methylene blue at 1 and 10 μM concentrations, respectively, that inhibited the endotoxin-induced increase in the levels nitrite, did not changed the endotoxin-induced levels of MDA. The levels of MDA in the presence of indomethacin or 3-aminobenzamide at 30 and 10 μM concentrations, that caused more significant inhibition on the endotoxin-induced increase in the levels of nitrite, were lower than the values of control and endotoxin (**P** < 0.05). The levels induced by these agents alone at the concentrations, that inhibited the endotoxin-induced increase in nitrite levels, were not different from control values (data not shown).

DISCUSSION

Using a whole blood and organ culture technique, we have shown that activation of iNOS, assessed by measuring the accumulation of the nitrite in the culture medium inhibited by selective iNOS inhibitor aminoguanidine, requires different endotoxin concentrations in whole blood, kidney and heart. Moreover, these findings indicate that blockade of GC, COX or PARS pathways decreases, increases or does not change the levels of nitrite depending on degree of inhibition and tissue type and iNOS-derived NO seems to decrease eicosanoid production via activation of GC or PARS enzymes.

In the present study an observation that is consistent with previous studies in isolated cells in culture (18,19), incubation of blood and kidney under unstimulated conditions caused increased levels during 48 h. This increase of nitrite may derive from cNOS, protein catabolism, NO derived from cytochrome P450 pathway or from tissue nitrite (20,21). It has also been demonstrated that the constitutive expression of iNOS in rat tissues (22).

It has been shown that the induction of NOS by endotoxin in many tissues and organs from endotoxemic animals is relatively short-lasting, with a time course which starts typically 2 h following endotoxin administration, peaks between 6-12 h and is largely over by 24 h (23,24). In the present study, although endotoxin increased the levels of nitrite in blood and organs, the concentrations of endotoxin to increase the levels of nitrite were different. On the other hand, the incubation period with endotoxin was not different in blood, kidney and heart. The findings are in agreement with previous studies reporting that there is a wide distribution of iNOS in tissues from endotoxemic animals (14,15). These results also show that the different levels of nitrite may indicate different percentages of cells capable of expressing iNOS within each organ and blood cells. The results with iNOS inhibitor, aminoguanidin, (3) support to these conclusions. In this study, aminoguanidine inhibited the increase in the levels of nitrite produced by kidney, heart and blood cells after endotoxin pretreatment. These results indicate that the NO detected as nitrite in the culture medium is formed by iNOS from L-arginine (25,26).

Methylene blue at the concentrations ranging from 0.001-10 μM also decreased the levels of nitrite elevated by endotoxin in these organs and blood cells. On the other hand, nitrite accumulation in the blood culture medium was increased by 10 μM methylene blue. In the present study, cGMP levels were not measured, however, the decrease in cGMP accumulation produced by methylene blue is generally accepted an indicating possible involvement of the NO-cGMP pathway in

several conditions. Methylene blue has been used an inhibitor of soluble GC even though it shows little specifity and, for example, interferes with NO formation. Since methylene blue has also been shown to be a generator of peroxynitrite (27) that produces nitrite, methylene blue at certain concentrations may increase the nitrite accumulation in the medium by this mechanism. Consistently with our findings, selective GC inhibitor ODQ has been reported not to decrease NO production, but it significantly increases NO levels in the presence of lipopolysaccharide plus interferon-γ in rat aortic smooth muscle culture indicating a potential negative feedback control by cGMP of the process of iNOS induction (28). The enhancement of the levels of nitrite by methylene blue seen in our study is in contrast to the data by Inoue et al. (29), in which exogenous or endogenous cGMP is able to enhance the expression of iNOS in human pulmonary smooth muscle cells in response to interleukin-1 stimulation. It is possible that the role of cGMP in the process of iNOS induction is dependent on the stimulus of iNOS induction, the cell type studied and also degree of inhibition of GC as seen with methylene blue. Based on previous findings and current results, it can be suggested that methylene blue decreases or increases the levels of nitrite via its direct (inhibition of iNOS) or indirect (inhibition of GS) effects on iNOS.

Indomethacin, nonselective COX inhibitor, also decreased the endotoxin-induced increase in the levels of nitrite in kidney and heart at the concentrations ranging between 0.1 and 300 μM. At 0.001-100 μM concentrations, indomethacin had no effect on the endotoxin-induced increase in the levels of nitrite in blood culture. There is mounting evidence for a direct link between NO production and synthesis of eicosanoids (10,11). NOS inhibitors act to increase COX expression and prostaglandin synthesis in response to cytokine stimulation and the addition of NO donors reverses the effect suggesting that NO also inhibits COX activity (30-32). NO has also shown to activates COX-1, but inhibits COX-2-derived prostaglandin production in vitro (33). Contrasting data have also been reported concerning the action of prostaglandins on the L-arginine/NO pathway. It has been shown that prostaglandins inhibit (34,35), increase (4,7,6,16,34,35) or do not affect (36) NO production by iNOS in several in vitro and in vivo models. This wide variability in the observations may result from the use of different cell lines and tissues, as well as differences in methods of cell activation or in inflammatory models (10,11). Milano et al. (35) demonstrated that exogenous prostaglandin E_2 at concentrations between 1 and 10 ng/ml was able to stimulate the expression of iNOS and the release of NO, while its higher concentrations (> 50 ng/ml) were inhibitory. Our present results with indomethacin showed that it is able to decrease or not change the nitrite levels depending on tissue and concentrations used. Based on the results of previous *in vitro* studies (35) and our present findings, it can be concluded that eicosanoids produced by COX at concentrations between 1-10 ng/ml appear to exacerbate the overproduction of NO by iNOS. We also measured the levels of TxB_2, as an index for COX activity, in the culture medium (6,37,38). Although endotoxin co-induces iNOS and COX-2 in a variety of cells, increased levels of NO by endotoxin was paralleled with a reduction in the levels of TxB_2 in blood and kidney, but not in the heart. Moreover, indomethacin further decreased the endotoxin-induced levels of TxB_2 in kidney. On the other hand, aminoguanidine increased the levels of TxB_2 in blood, but not in kidney. These results confirm previous findings reporting that NO produced by iNOS inhibits COX (30,32,33).

PARS is a nuclear enzyme which, when activated by DNA strand breakage which can be induced by a variety of environmental stimuli and free radical attacks, initiates DNA injury and PARS activation (8,38). It appears that PARS also plays an important role in regulating the expression of a variety of genes including iNOS. Considering the potential importance of PARS activation during NO-mediated apoptosis and the role of peroxynitrite, a potent oxidant formed by the reaction of O_2^- and NO, the current study was designed to investigate the effect of pharmacological blockade of PARS by 3-aminobenzamide on the endotoxin-induced increase in the levels of nitrite derived from NO by iNOS. Accumulation of nitrite in the culture medium was inhibited by selective PARS inhibitor, 3-aminobenzamide at 0.1-1000 μM concentrations in the kidney and heart. On the other hand, 3-aminobenzamide at 30 μM increased the levels of nitrite in kidney culture. The endotoxin-induced levels of nitrite in blood were not affected by 3-aminobenzamide at 0.001-10 μM concentrations. Based on these observations, it can be concluded that inhibition of PARS may increase or decrease the levels of nitrite derived from NO by iNOS depending on tissue.

In the present study, although levels of nitrite was increased after endotoxin pretreatment, there was no change in the levels of MDA in kidney and heart culture medium. This finding supports the inhibitory effect of NO on lipid peroxidation (9,39,40). On the other hand, indomethacin and 3-aminobenzamide at concentrations that inhibit endotoxin-induced increase in nitrite levels, decreased the endotoxin-induced levels of MDA only in the heart. It can be concluded that since indomethacin also decreased the endotoxin-induced levels of TxB_2 as well as levels of nitrite in the heart, some of COX products may have an inhibitory effect on lipid peroxidation. Although peroxynitrite levels were not measured in this study, the results with 3-aminobenzamide may suggest an inhibitory role of peroxynitrite on lipid peroxidation. Since aminoguanidine prevents the induction of iNOS and possesses direct scavenging properties against free radicals and peroxynitrite, it is not surprising that aminoguanidine affects lipid peroxidation. Results obtained from the effects of aminoguanidine and methylene blue on MDA production also support the prooxidant property of NO on lipid peroxidation.

CONCLUSION

In summary, our results showed that endotoxin caused a differential induction of NO production by iNOS in whole blood, kidney and heart depending on its concentration. The inhibitory effects of aminoguanidine, methylene blue, indomethacin or 3-aminobenzamide on the endotoxin-induced increase in nitrite levels seem to be dependent on the inhibition of iNOS through direct or indirect mechanisms. The differential effects of endotoxin and/or the inhibitors may also depend on the individual contribution of several cell types in these tissues. Moreover, iNOS-derived NO seems to decrease eicosanoid production via activation of GC or PARS enzymes. These results emphasise the important role of iNOS, GS, COX and/or PARS pathways and the interactions between them in the dysfunction or failure of major organs and circulating blood cells in response to endotoxin.

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