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An increase in nitric oxide produced by rat peritoneal neutrophils is not involved in cell apoptosis

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POLYMORPHONUCLEAR neutrophils (PMN) obtained from carrageenin-stimulated peritoneal cavities of rats, but not blood PMN, spontaneously produced nitric oxide (NO) when incubated *in vitro*. Incubation of the cells with the NO synthase inhibitors, L-imino-ethyl-L-ornithine (L-NIO) or N^G-monomethyl-L-arginine (L-NMMA), inhibited NO production. This inhibition could be reversed by L-arginine. Incubation of PMN with lipopolysaccharide (LPS) failed to enhance NO production. Pretreatment of the rats with dexamethasone (DEXA) prior to carrageenin injection or incubation of PMN with the glucocorticoid *in vitro* partially inhibited the spontaneous release of NO. On the other hand, when PMN obtained from DEXA pretreated rats were incubated *in vitro* with DEXA, NO synthase activity and hence NO generation were almost abolished. A similar inhibition was also observed following the addition of L-NIO or cycloheximide to cultures of carrageenin-elicited PMN. The NO production by PMN did not appear to be related to cell viability or apoptosis. Indeed, neither the blockade of NO generation by L-NIO nor the incubation of the neutrophils with a NO donor, *S*-nitroso-acetyl-penicillamine (SNAP) modified the pattern of LDH release or DNA fragmentation. In summary, it appears that PMN migration triggers a continuous NO synthesis, and that NO produced by these cells is not related to their apoptosis.

Key words: Inflammation, Nitric oxide synthase, Programmed cell death.

Introduction

Eukaryotic cells die either by necrosis caused by different kinds of injurious stimuli or by apoptosis, a process of self-destruction which is genetically programmed. This programmed cell death is a mechanism responsible for the removal of redundant cells during embryogenesis[1] and organ involution,[2] and seems to play a controlling role in counteracting cell proliferation.[3] Ongoing apoptosis is characterized by specific alterations in the cell's morphology, including condensation of the nuclear chromatin.[4] This event seems to result from the activation of an endogenous endonuclease which cleaves DNA into fragments that are multiples of 180–120 bp.[5]

Nitric oxide (NO) has been implicated in various physiological and pathological processes including cell injury and apoptosis.[6,7] NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by the enzyme NO synthase (NOS), of which two general classes have been identified. Constitutive NO synthase (cNOS) is a Ca^{2+}/calmodulin- and NADPH-dependent enzyme, present in vascular endothelium, brain and platelets.[8] The cNOS is mainly involved in physiological processes in the cardiovascular and nervous systems.[9] Inducible NO synthase (iNOS) can be induced by LPS and cytokines in a large variety of cells including macrophages, hepatocytes, and vascular smooth muscle and endothelial cells.[10] In contrast to cNOS, the activity of iNOS is Ca^{2+}-independent and its induction is inhibited by glucocorticoids.[11–13] The microbicidal and tumouricidal activities of macrophages are dependent on the generation of large amounts of NO resulting from the expression of iNOS in these cells.[14,15]

It has been shown that NO is involved in macrophage apoptosis.[6,7] On the other hand, although neutrophils undergo extensive apoptosis at the inflammatory site,[16] the role of NO in this process has not been addressed. Whereas NO production by macrophages is brought about only if the cells are stimulated with LPS and/or some cytokines,[17] it has been reported that elicited neutrophils were able to produce

NO spontaneously.[18] This production was not enhanced by LPS or cytokines.[19] It appears that the induction of NOS in neutrophils may be triggered by cell migration to the inflammatory site.[18,19] The properties of neutrophil NOS are still not well known, although it seems to be an unique enzyme.[20] It is also unclear whether a turnover of NOS *in vitro* would be responsible for the continuous NO production by elicited neutrophils.[11,18]

In the present paper, we have studied the kinetics of NO production by carrageenin elicited-neutrophils, and we provide some evidence that a continuous turnover of NO synthase does occur *in vitro*. In addition, experiments were conducted to investigate the involvement of neutrophil-generated NO in the process of apoptosis of these cells.

Materials and Methods

Animals: Male albino Wistar rats, weighing between 150 – 200 g, were maintained in temperature-controlled rooms at 23 – 25°C, with free access to food and water.

Peritoneal polymorphonuclear neutrophil preparation: Rat peritoneal cavities were stimulated by the injection of carrageenin (300 µg/cavity). Four and 12 h later, the peritoneal cells were harvested in phosphate buffered saline (PBS). The exudate cells were centrifuged (200 × ***g***, 10 min) and resuspended in RPMI-1640 medium (Sigma, USA). In order to remove the macrophages, the cell suspension was allowed to adhere to plastic tissue culture dishes for 1 h at 37°C in a CO_2 incubator. The erythrocytes present in the non-adherent cells were removed by hyposmotic lysis, and polymorphonuclear neutrophils (PMN) were resuspended at a final concentration of 5×10^6 cells/ml of RPMI supplemented with 10% of fetal calf serum (FCS; Gibco, USA) and 100 U/ml of penicillin (Sigma, USA). The PMN content of the suspension was 95%. The cell viability was assessed by their ability to exclude trypan blue dye. In some experiments, the animals were pretreated with dexamethasone (Sigma, USA; 1 mg/kg, s.c.) 6 h and 30 min prior to, or together with, L-nitroarginine (Wellcome, UK; 30 mg/kg, s.c.) 30 min before the administration of carrageenin.

Peripheral blood neutrophils: Rats were anaesthetized with ether and the blood collected by cardiac puncture in plastic syringes containing heparin. Leucocytes were isolated by dextran sedimentation, followed by hypotonic lysis. Blood PMN were resuspended at a final concentration 10^6 cells/ml RPMI supplemented with FCS and penicillin. The cell viability was assessed by the exclusion of trypan blue dye.

PMN incubation in vitro*:* One ml aliquots of peritoneal or blood PMN suspended in RPMI medium were drawn into sterile Eppendorf tubes and incubated for 1 h to 72 h at 37°C. In some experiments, the following drugs were added to the cell suspension at the beginning of the incubation: L-NMMA (Wellcome, UK; 200 µM), L-NIO (Wellcome, UK; 200 µM), L-arginine (Sigma, USA; 2 mM), dexamethasone (10 µM), cycloheximide (Sigma, USA; 10 µM), LPS (lipopolysaccharide from *Escherichia coli* 0111.84, Sigma, USA; 1 µg/ml) or SNAP (Wellcome, UK; 1 mM). At selected times, the PMN suspensions were centrifuged (2000 × ***g***, 5 min) and the supernatants stored at −20°C until measurement of the nitrite content. In some experiments, the NOS activity of the cell pellet was also determined.

Nitrite assay: Nitrite levels were measured by the colorimetric assay based on the Griess reaction.[21] Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in 5% phosphoric acid) was added to an equal volume of the supernatants and the absorbance at 546 nm measured after 10 min. The nitrite (NO_2^-) concentration was determined by reference to a standard curve of sodium nitrite.

NOS activity assay: At indicated times, PMN were sonicated in the extraction buffer (320 mM sucrose, 50 mM TRIS, 1 mM dithiotreitol, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor and 2 µg/ml aprotinin) and centrifuged (10 000 × ***g***) for 20 min at 4°C. NOS activity in the supernatant was determined based on the conversion of L-[U-^{14}C] arginine (20 µM) to L-[U-^{14}C] citrulline following a 20 min incubation at 37°C as described previously.[22] Unlabelled L-citrulline (1 mM) and L-valine (50 mM) were also added to the assay in order to inhibit arginase activity.[22] The protein content of the supernatants was determined by the Coomassie blue binding method according to the manufacturer's recommendations (Pierce Chemical, Rockford, IL). Total NOS activity was expressed as pmol citrulline formed/mg protein/min.

Measurement of lactate dehydrogenase: The release of cytoplasmic lactate dehydrogenase (LDH) into the medium was used to evaluate PMN viability. The enzyme activity was measured by the assay based on the LDH-induced reduction of pyruvate to lactate in the presence of NADH, as described previously.[23] Briefly, 0.1 ml

of fresh PMN supernatants obtained as described above (free of red blood cell contamination) were added to spectrophotometer cuvettes containing 2 mM of NADH and 10 mM of sodium pyruvate. The decrease in the absorbance at 340 nm was followed for 4 min (enzymatic rate of LDH). Each sample was run in parallel with a positive control (cells disrupted with 0.2% digitonin) considered as 100% of death. LDH activity in PMN supernatants was expressed as a percentage of the positive control.

Apoptosis assays: After the indicated times, the PMN suspensions were centrifuged (2 000 × **g**, 5 min), the supernatants were removed and the pelleted cells were lysed with 0.5 ml of lysing buffer (0.2% Triton X-100, 10 mM Tris and 1 mM EDTA, pH 7.4). The tubes were then centrifuged for 10 min at 13 000 × **g** and the supernatants were immediately separated. Half the volume was used for gel electrophoresis and the other half, as well as the pellet, was used for the diphenylamine (DPA) assay as described below.

Gel electrophoresis: The supernatant obtained as described above was treated with 0.3 ml 5 M NaCl and isopropanol and left for 12 h at −70°C. DNA pellets were obtained by centrifugation (13 000 × **g**, 10 min), washed twice with 70% ethanol, air dried, resuspended in 10 mM Tris, 1 mM EDTA (pH 7.4) and incubated at 37°C for 10 min. A loading buffer (20% Ficoll 400, 0.1 M EDTA, 1% SDS, O.25% bromophenol blue) was added at a final proportion of 10:1 and the samples were electrophoresed on 1% agarose gel containing 1 µg of ethidium bromide/ml. The DNA bands were visualized and photographed under UV illumination.

DPA reaction: Two hundred and fifty µl of trichloroacetic acid (TCA, 25%) were added to the pellet containing uncut DNA and to the other half of the supernatant containing DNA fragments. The tubes were incubated overnight at 4°C and the precipitated DNA was pelleted by centrifugation (10 min, 13 000 × **g**). Eighty µl of a 5% TCA solution was added to each pellet and DNA was hydrolysed by heating for 15 min at 90°C. One hundred and sixty µl of a freshly preparated DPA reagent (150 mg DPA, 10 ml glacial acetic acid, 0.15 ml concentrated sulfuric acid, 0.05 ml 1.6% acetaldehyde solution) were then added to the tubes and the colour was allowed to develop for 4 h at 37°C. The reaction was quantitated spectrophotometrically at 570 nm. The percentage of fragmented DNA was calculated using the formula

$$\% \text{ fragmented DNA} = \frac{\text{OD (SN)}}{\text{OD (SN)} + \text{OD (pellet)}} \times 100$$

where SN is the supernatant and OD is the optical density.

Statistical analysis: Statistical significance was assessed by ANOVA followed by the Bonferroni t test and $p < 0.05$ was taken as statistically significant.

Results

Production of NO_2^- by rat peritoneal PMN: Neutrophils harvested from rat peritoneal cavities stimulated with carrageenin spontaneously release NO_2^- when incubated *in vitro*. The levels of NO_2^- in the medium were already detectable after 1 h of incubation and increased continuously over 24 h. The cells harvested from peritoneal cavities 12 h after the carrageenin injection released about 4-fold more NO_2^- than those obtained 4 h post-injection (Fig. 1(A)). The addition of LPS (1 µg/ml) to the PMN suspension did not modify the pattern of NO_2^- accumulation in the supernatants. Fig. 1(B) shows that NO_2^- generation by PMN was abolished by the presence of 200 µM L-NIO (the NO_2 concentration was equivalent to that measured in the culture medium) and was partially inhibited by L-NMMA at the same concentration. The L-NMMA effect was completely reversed by L-arginine (2 mM).

Production of NO_2^- by rat peritoneal blood PMN: In contrast to peritoneal neutrophils, blood PMN collected from untreated or carrageenin-stimulated rats, did not produce significant amounts of NO_2^-, after a 24 h incubation *in vitro*, spontaneously or even after stimulation with LPS (10 µg/ml) *in vitro*. Results for untreated rats were 1.5 ± 1.4 µM (controls) and 2.3 ± 2.0 µM (LPS stimulated PMN); while results for carrageenin treated rats were 2.9 ± 2.2 µM (controls) and 6.5 ± 4.7 µM of NO_2 (LPS stimulated PMN).

Effect of dexamethasone, L-NIO, and cycloheximide on NO_2^- production and NOS activity of peritoneal neutrophils: Fig. 2 shows that the pretreatment of the rats with dexamethasone prior to carrageenin injection partially inhibited the spontaneous release of NO_2^- by peritoneal neutrophils. A similar inhibition was obtained when neutrophils were treated *in vitro* with dexamethasone. However, the addition of dexamethasone (10 µM) *in vitro* to PMN obtained from dexamethasone-pretreated rats strongly inhibited NO_2^- generation. The same effect was

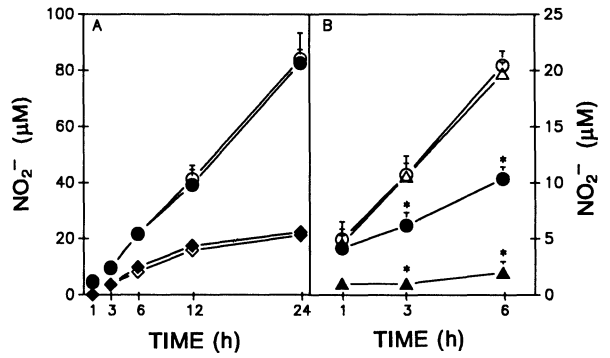


FIG. 1. (A) The time course and effect of LPS (1 μg/ml; filled symbols) on NO_2^- generation by cultured rat PMN harvested from the peritoneal cavity 4 h (◆) and 12 h (●) after carrageenin (Cg) stimulation. The open symbols represent the response in the absence of LPS. (B) The inhibitory effect of 200 μM L-NMMA (●) and 200 μM L-NIO (▲) on NO_2^- generation by PMN harvested 12 h after Cg. L-Arginine (2 mM,△) reversed the inhibitory effect of L-NMMA. The open circle represents the response in the absence of NOS inhibitor. Each point is the mean ± S.D. ($n=5$). *$p < 0.05$ compared to non-treated group (ANOVA, followed by Bonferroni's t test).

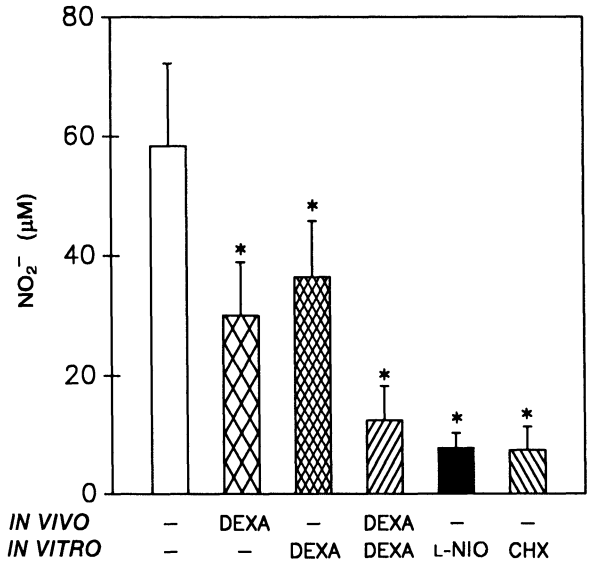


FIG. 2. The *in vitro* effect of dexamethasone (DEXA, 10 μM), L-NIO (200 μM) and cycloheximide (CHX, 10 μM) on NO_2^- generation by PMN harvested from the Cg-injected peritoneal cavity of untreated (–) rats or from animals pretreated with DEXA (1 mg/kg). The cells were harvested from rats injected 12 h previously with carrageenin and were incubated for 24 h *in vitro*. The bars represent the mean ± S.D. ($n=5$). *$p < 0.05$ compared to non-treated group (ANOVA followed by Bonferroni's t test).

observed when L-NIO (200 μM) and cycloheximide (10 μM) were incubated *in vitro* with neutrophils obtained from untreated rats.

In agreement with these results, the NOS activity assayed in neutrophils treated *in vitro* or *in vivo* with dexamethasone was inhibited when compared with control cells: control, 2.30 ± 0.30; dexamethasone *in vivo*, 0.78 ± 0.08 and dexamethasone *in vitro*, 0.46 ± 0.05 pmol of citrulline formed/min/mg protein.

Release of LDH and DNA fragmentation: Table I shows that there was no correlation between the levels of NO generated and the release of LDH by rat peritoneal PMN incubated for 24 h *in vitro*. The incubation of neutrophils obtained from untreated or nitro-arginine-pretreated rats with L-NIO blocked NO_2^- production but did not change the amount of LDH released. The same result was obtained when PMN were treated *in vitro* with dexamethasone. In addition, SNAP, an NO donor, even at a high concentration (1 mM), did not affect the LDH release. Smaller doses of SNAP (0.1 and 0.3 mM) also did not affect the LDH release.

The analysis of DNA fragmentation in PMN harvested 4 h after carrageenin injection (Fig. 3(A)), showed only 1 to 4% of fragmented DNA. When these cells were incubated for 24 h *in vitro*, in the presence or in the absence of L-NIO or SNAP, the percentage of segmented DNA increased to 23%. The same pattern of DNA fragmentation was also seen with PMN obtained from animals pretreated *in vivo* with dexamethasone (not shown in the figure). On the other hand, this percentage of fragmentation rose to about 60% when dexamethasone was added to the medium. Blood PMN, when incubated *in vitro* for 24 h, showed a similar pattern of DNA fragmentation ($t_0 = 8 \pm 2.4\%$; $t_{24} = 29.4 \pm 8.8\%$), although it did not produce NO.

The gel electrophoresis of DNA extracted from neutrophils incubated for 24 h (Fig. 3(B), Lane 3) showed, in contrast with non-incubated cells (Lane 2), the internucleosomal fragmentation pattern typical of endonuclease activation and apoptosis. The characteristic DNA ladder with 180-bp steps did not change, even when NO generation was inhibited by L-NIO or dexamethasone (Lanes 4 and 6). The addition of SNAP, an NO donor, at a dose of 1 mM, also did not change the pattern of DNA fragmentation (Lane 5). Smaller doses of SNAP (0.1 and 0.3 mM) gave similar results. As with LDH release, the index of DNA fragmentation was not altered in neutrophils obtained from rats pretreated with nitro-arginine and incubated *in vitro* for 24 h in the presence or absence of L-NIO.

Discussion

In the present investigation, we have shown that neutrophils obtained 4 and 12 h after i.p. injection of carrageenin continuously and spontaneously produced NO_2^- when incubated *in vitro*. This production was partially inhibited by L-NMMA or abolished by L-NIO. This inhibition could be reversed by L-arginine. These results clearly indicate that NO_2^- measured by the Griess

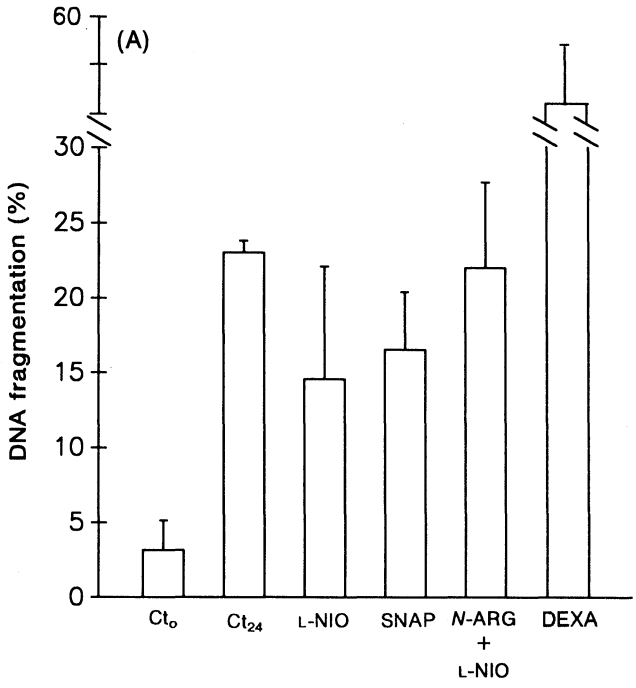
Table 1. Generation of NO_2^- and LDH release (% total) by rat peritoneal PMN incubated for 24 h *in vitro*

Treatment	LDH (%) ± S.D.		NO_2 (μM) ± S.D.	
	Cg, 4 h	Cg, 12 h	Cg, 4 h	Cg, 12 h
Control (Cg, i.p.)	18.2 ±3.0	36.0 ±2.0	23.4 ± 1.9	87.5 ± 6.0
Nitro-arg *in vivo* (30 mg/kg)	24.8 ± 3.1	35.0 ± 7.0	22.7 ± 1.7	89.7 ± 2.4
L-NIO *in vitro* (200 μM)	22.6 ± 1.71	29.0 ± 2.2	3.90 ± 1.0*	7.70 ± 2.6*
Nitro-arg *in vivo* + L-NIO *in vitro*	16.8 ± 6.0	35.0 ± 6.2	4.11 ± 1.2*	3.67 ± 2.8*
Dexamethasone *in vitro* (10 μM)	23.6 ± 2.7	33.82 ± 1.6	12.8 ± 2.7*	41.7 ± 4.8*
SNAP *in vitro* (1 mM)	24.2 ± 6.5	32.3 ± 3.8	ND	ND

ND: not determined. *$p < 0.05$ compared to control values (ANOVA followed by Bonferroni's t test).

method resulted from the oxidation of NO generated through the activity of the NOS present in neutrophils. It must be pointed out, however, that circulating neutrophils obtained from either untreated or carrageenin-stimulated rats did not synthesize NO in identical *in vitro* experimental conditions. These data support the idea[18] that the passage of neutrophils to the inflammatory site may be the mechanism responsible for the triggering of NOS activity. Cytokines produced at the onset of the inflammatory response may perhaps activate NOS in PMN which will then produce NO once they arrive at the inflammatory site. This suggestion is supported by the findings that NOS activity in PMN obtained from animals pretreated with dexamethasone, which has a known inhibitory effect on cytokine production[24] and on NOS induction, was partially but significantly inhibited. Furthermore, it appears that in the PMN the threshold level for NOS induction reaches its maximum in peritoneal cavities, since LPS at doses which usually stimulate iNOS in other cells, particularly in macrophages,[16] failed to further stimulate NOS in these PMN. The observations that cycloheximide, a protein synthesis inhibitor, blocked NO production *in vitro*, and that dexamethasone also partially inhibited the NO production *in vitro*, suggest that NO generation by elicited PMN *in vitro* also requires a continuous synthesis of NOS. These findings are in line with previous data,[11] but contrast with the absence of an affect of cycloheximide on the spontaneous release of NO_2^- by neutrophils obtained from rat peritoneal cavities challenged with oyster glycogen.[19] The activation of iNOS was further confirmed by the observation that the combined *in vivo* and *in vitro* treatment with dexamethasone almost abolished NO_2^- production and NOS activity, the latter measured by the citrulline assay.

Recently, it was shown that NO production appears to be associated with macrophage programmed cell death.[6,7] Our results indicate that in neutrophils there was no correlation between the loss of cell viability (as assessed by LDH release) or DNA fragmentation (an indicator of apoptosis) and NO production. This was shown in experiments in which L-NIO, at a concentration that abolished NO generation, or SNAP, a NO donor used at an effective microbicidal concentration,[25] failed to change the pattern of DNA fragmentation or of LDH release observed for neutrophils incubated for 24 h. In some experiments, the effects of endogenous NO were avoided by pretreating the animals with L-nitroarginine and the peritoneal cells were incubated *in vitro* with L-NIO. Although these treatments completely prevented NO generation, the pattern of DNA fragmentation or LDH release did not change. In agreement, non-stimulated blood neutrophils which did not release NO, also underwent apoptosis after 24 h of incubation *in vitro*. Moreover, pretreatment of the rats with dexamethasone, which was shown to inhibit the induction of NOS *in vivo*, did not decrease DNA fragmentation observed after 24 h of PMN incubation (data not shown). On the other hand, when dexamethasone was added *in vitro* to PMN suspensions an enhancement of apoptosis occurred, an effect already observed for thymocytes[26] and T cell leukaemia lines.[27] This effect of dexamethasone is probably not related to its action in inhibiting NOS. Taken together, our data indicate that NO produced by rat peritoneal neutrophils



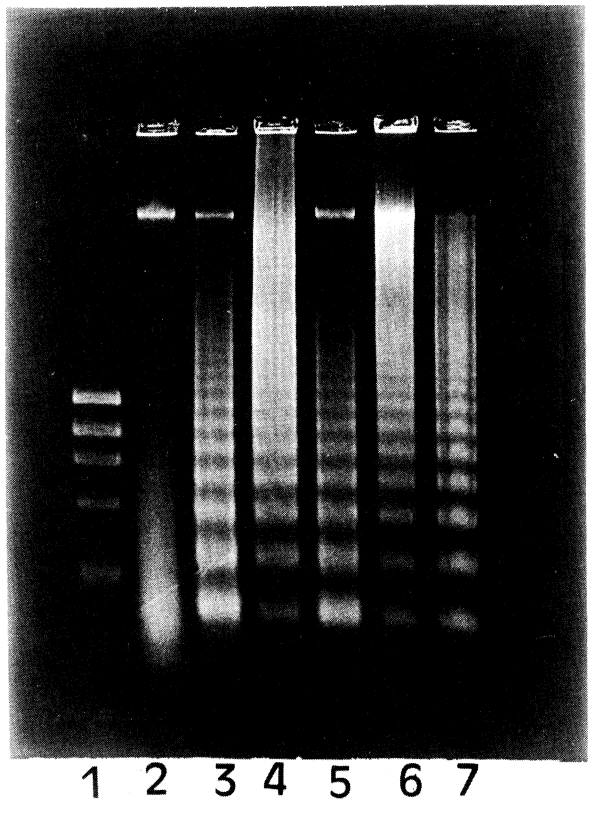


FIG. 3. (A) The *in vitro* effect of different treatments on DNA fragmentation. PMN were cultured for 24 h with the indicated reagents: RPMI (Ct_{24}) L-NIO (200 μM), SNAP (1 mM), and dexamethasone (DEXA, 10 μM). PMN from rats pretreated with L-nitroarginine (30 mg/kg) were also cultured with L-NIO (200 μM; *N*—Arg+L-NIO). The percent of DNA fragmentation was assayed by the DPA method. The first bar (Ct_0) shows the percentage of DNA fragmentation of the non-incubated PMN. The data are the mean ± S.D. of five determinations from a representative experiment. (B) Gel electrophoresis of DNA isolated from PMN before any incubation (Lane 2) and from PMN cultured for 24 h without (Lane 3) or with L-NIO, SNAP or dexamethasone (Lanes 4–6, respectively). The first lane shows the molecular weight markers.

is not involved in the apoptosis of these cells *in vitro*. Further investigation is required in order to establish which mediators are involved in this process.

In summary, we have shown here that PMN migration to the inflammatory site probably triggers NOS induction and that NO generation at the inflammatory site is not associated with the phenomenon of apoptosis in these cells.

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