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NITRIC OXIDE-DEPENDENT N-NITROSATING ACTIVITY OF RAT PLEURAL MESOTHELIAL CELLS

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Recent studies have demonstrated that nitric oxide (NO)-derived N-nitrosating agents may promote mutagenesis and carcinogenesis from the nitrosative deamination of DNA bases via the formation of nitrosamine intermediates. The objective of this study was to determine if pleural mesothelial cells (PMC) stimulated with proinflammatory cytokines could promote the N-nitrosation of a primary aromatic amine via the L-arginine-dependent formation of NO-derived N-nitrosating agents. N-nitrosating activity was determined by measuring the N-nitrosation of a model amine, 2,3-diaminonapthalene, to yield its fluorescent triazole (1-naptho-2,3-triazole) derivative. Results show that specific combinations of TNF, IL-1, interferon gamma, and LPS significantly increased N-nitrosating activity. There was a significant positive correlation between nitrite plus nitrate and triazole production. Triazole formation was inhibited by N^G-nitro-L-arginine methyl ester, suggesting that triazole was derived from the L-arginine-dependent formation of NO. These data indicate that PMC have the capacity to promote the N-nitrosation of primary aromatic amines via the formation of NO.

KEY WORDS: pleura, inflammation, tumor necrosis factor, interleukin-1, nitrosamine

ABBREVIATIONS: pleural mesothelial cell(s), PMC; nitric oxide, NO; tumor necrosis factor-alpha, TNF; interleukin-1-beta, IL-1; interferon gamma, IFN; lipopolysaccharide, LPS; nitrogen dioxide, NO_2; dinitrogen trioxide, N_2O_3; dinitrogen tetroxide, N_2O_4; N^G-nitro-L-arginine methyl ester, L-NAME; Dulbecco's modified Eagle medium, DMEM; 2,3-diaminonaphthalene, DAN; 1-naphtho(2,3)-triazole, NAT.

INTRODUCTION

The pleura consists of a single layer of mesothelial cells which rest on a layer of connective tissue that is primarily composed of collagen and elastin. Because of their location, pleural mesothelial cells (PMC) are the first cells to encounter foreign bodies, cells, and other substances entering the pleural space and are therefore in a prime position to react to and regulate processes in the pleura and pleural space. Inhalation of asbestos fibers leads to eventual deposition of the fibers in the peripheral lung and pleura with the subsequent accumulation of macrophages on the pleural surface (1). Exposure of macrophages to asbestos results in the production of a variety of cytokines, including tumor necrosis factor, interferon gamma and interleukin-1 (2–5). While

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transformation of mesothelial cells into mesothelioma cells can occur after asbestos-induced injury and inflammation, the mechanism of this alteration remains unclear.

Nitric oxide (NO) is a free radical derived from the terminal guanidino nitrogen of L-arginine by the action of NO synthase (6). It is unstable in the presence of molecular oxygen and will rapidly and spontaneously react to yield a variety of nitrogen oxides such as nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3), as well as, the stable anions nitrite and nitrate (7). It is known that N_2O_3 is a potent nitrosating agent that may react with a variety of primary and secondary amines to yield potentially mutagenic and carcinogenic nitrosamines (7).

Although it has been demonstrated that activated macrophages and neutrophils are capable of producing nitroso derivatives of certain amines *in vitro*, the ability of PMC to synthesize these potential carcinogens has not been investigated (7, 8). We have previously shown that rat PMC exposed *in vitro* to proinflammatory cytokines and lipopolysaccharide (LPS) can produce large amounts of NO via the stimulation of the inducible isoform of nitric oxide synthase (9, 10). PMC production is on a level equal to that of macrophages and type II pneumocytes (> 100 nanomoles/10^6 cells/72 h) (11, 12). We hypothesize that enhanced PMC reactive nitrogen metabolism and N-nitrosating activity may represent a link between chronic asbestos-induced pleural inflammation and malignant transformation of PMC *in vivo*. The objective of this study was to determine if PMC stimulated *in vitro* with proinflammatory cytokines and LPS could promote the N-nitrosation of a primary aromatic amine in a manner dependent on the formation of NO.

MATERIALS AND METHODS

Source of Materials

Human recombinant TNF-alpha (10^7 units/mg protein) was obtained from Amgen (Thousand Oaks, CA), murine recombinant interleukin-1-beta (3.5×10^5 units/mg protein) was obtained from Genzyme (Cambridge, MA), and rat interferon gamma (4×10^6 units/mg protein) was obtained from Gibco (Gaithersburg, MD). Ham's F-12 medium, phenol red-free Dulbecco's modified Eagle medium (DMEM) and dialyzed fetal bovine serum (FBS) were purchased from Gibco (Grand Island, LPS from *Escherichia coli* was obtained from Difco Laboratories (Detroit, MI). Sulfanilamide, naphthalene diamine dihydrochloride, 2,3-diaminonapthalene and N^G-nitro-L- arginine methyl ester (L-NAME) were obtained from Sigma (St Louis, MO), as were all other chemical reagents.

Cell Culture

Rat PMC (line 4/4 RM-4) derived from the visceral pleura of a female Fischer 344 rat of the Charles River strain were obtained from the American Type Culture Collection (Rockville, MD). This cell line has been previously identified as a mesothelial cell line by its cobblestone morphology and characteristic staining for hyaluronic acid, keratin and vimentin (13). The cells were routinely grown in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, glutamine (2 mM) and gentamicin (50 ug/ml) (subsequently referred to as complete medium) in a 95% air:5% CO_2, humidified atmosphere. Cells were split weekly 1:3 by trypsinization. Passages 2–10 were used for these experiments.

N-nitrosating Activity

The ability of quiescent or cytokine-stimulated PMC to N-nitrosate a model aromatic amine (2,3-diaminonaphthalene) was quantified as described below. Once confluent, media were changed to Earle's salts containing 20 mmol/L HEPES (pH 7.4), 20 mmol/L glucose, 4 mmol/L glutamine, 1 mmol/L L-arginine, 50 ug/ml gentamicin and 10% FBS. PMC were then incubated with triple combinations of TNF (50 ng/ml), IL-1-beta (100 ng/ml), IFN gamma (500 units/ml), and LPS (50 ug/ml) or control medium (no cytokines or LPS) for 72 hours. 2,3-diaminonapthalene (DAN) is a primary aromatic diamino compound that can be N-nitrosated to yield its fluorescent triazole derivative, 1-naphtho(2,3)triazole (NAT) (14). DAN was included at a concentration of 0.2 mmol/L to detect N-nitrosating activity. For some experiments the NO synthase inhibitor L-NAME (1 mmol/L) was included. When appropriate the inhibitory effect of L-NAME was confirmed by the reversal of this effect with L-arginine (5 mmol/L). After incubation supernatants were removed, centrifuged and placed into glass tubes. To the 0.5 ml supernatants, 2.5 ml of 10 mM NaOH was added, and the fluorescence was determined using an excitation wavelength of 375 nm and an emission wavelength of 450 nm (14). Concentrations of the triazole derivative (NAT) were calculated from a standard plot using known concentrations of the purified triazole nitrosamine synthesized as previously described by Wheeler *et al.* (15). The cell layers were washed with PBS, trypsinized, and cell number determined by direct cell counts. Results were expressed per 10^6 cells.

Nitrite and Nitrate

Nitrite plus nitrate were measured as previously described (9). Briefly, the supernatant was removed from each well following incubation, centrifuged to remove cellular debris, and placed in tubes for nitrite plus nitrate determination. Nitrate was reduced to nitrite using bacterial nitrate reductase. The source of our nitrate reductase in these experiments was *Escherichia coli* (ATCC #25922, American Type Culture Collection, Rockville, MD). Nitrate was reduced to nitrite by incubating 0.5 ml of supernatants with 0.050 ml of baterial suspension for 60 minutes at 37°C. After centrifugation at 10,000×g for 5 minutes at 25°C, supernatants were incubated with 0.5 ml of Griess reagent (0.1% naphthalene diamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4), incubated for 10 minutes at 25°C and the absorbance at 543 nm was measured. Nitrate was calculated from a standard curve using sodium nitrate reduced by *E. coli* as the standard.

Statistical Analysis

Data is expressed as mean±standard deviation. The significance of difference in nitrosamine concentrations between experimental groups was determined by analysis of variance. When analysis of variance indicated a significant difference between groups, a t test within the analysis of variance was done to determine which intergroup differences were significant. Statistical significance was defined as a p value less than 0.05.

RESULTS

The N-nitrosation of 2,3-diaminonapthalene to yield 1-naptho-2,3-triazole was significantly increased by exposure of PMC to triple combinations of TNF, IL-1, IFN and LPS (Figure 1). The concentrations of agents and time of exposure used were those that previously were shown to cause maximal NO production by the PMC (9). These factors were chosen since they have been found in pleural effusions and pleural and lung tissue during a variety of inflammatory conditions associated with pleural disease (2–5, 16, 17). The individual cytokines or LPS alone did not significantly affect triazole production (in parallel with their lack of an effect on PMC NO synthesis, 9). The combination of IL-1, TNF and LPS caused the highest level of triazole production (1,458±71 picomoles/10^6 cells), whereas triazole production by control (unstimulated) PMC was 20±3 picomoles/10^6 cells. There was also a positive correlation between triazole formation and nitrite plus nitrate production with the combination that gave the highest nitrite plus nitrate production also giving the highest nitrosating activity

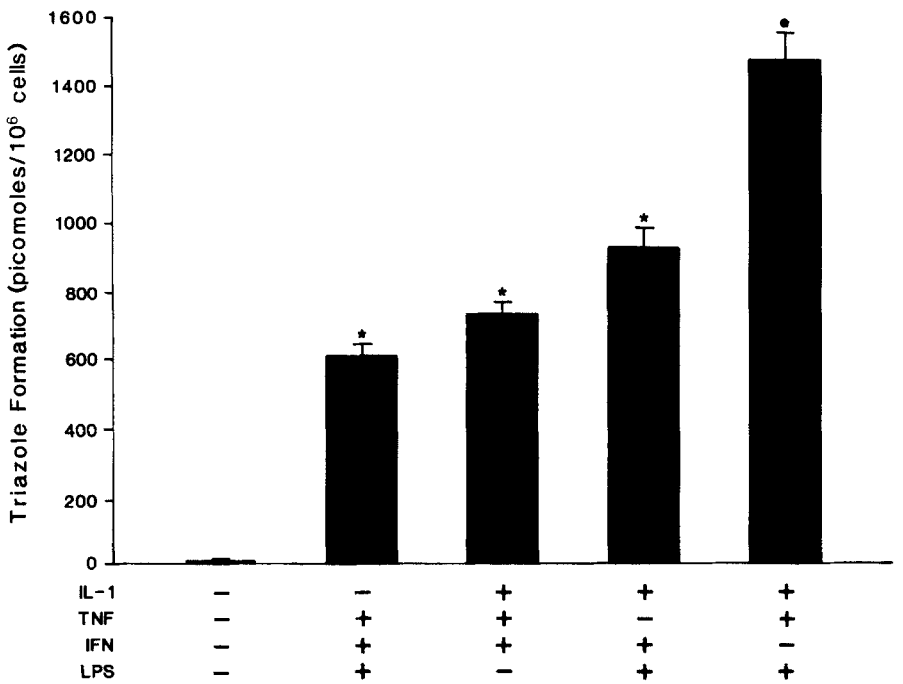

FIGURE 1 N-nitrosating activity of confluent monolayers of rat pleural mesothelial cells stimulated with triple combinations of LPS (50 ug/ml), IFN-gamma (500 units/ml), TNF (50 ng/ml), or IL-1 (100 ng/ml) for 72 hours. Production of the highly fluorescent triazole derivative, 1-naphtho(2,3)triazole, of 2,3-diaminonaphthalene was quantified and used as an index of N-nitrosating activity as described in MATERIALS AND METHODS. Results are expressed as mean±standard deviation corrected for control (n = 4). Three separate experiments yielded similar results. *P<0.05 vs. control.

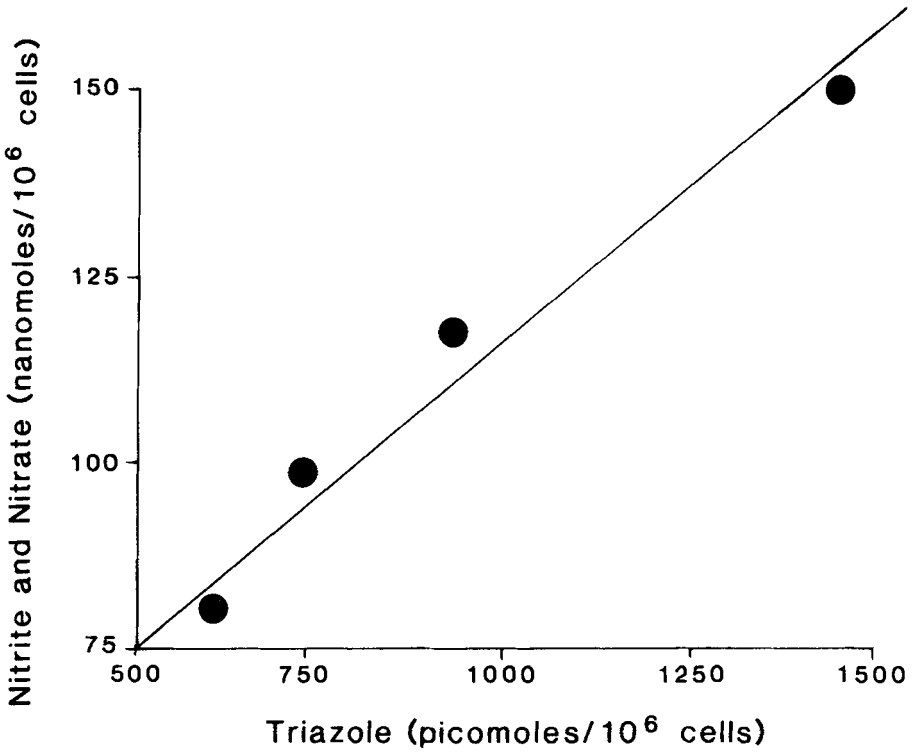

FIGURE 2 Correlation of triazole formation with nitrite plus nitrate production. Three separate experiments yielded similar results ($r = 0.98$, $p = 0.02$).

($r = 0.98$, $p = 0.02$) (Figure 2). The ratio of triazole to total nitrite plus nitrate was approximately 1%. Triazole formation was significantly inhibited by L-NAME (a competitive inhibitor of nitric oxide synthase) and recovered by the addition of L-arginine, suggesting that the N-nitrosating agents were derived from the L-arginine-dependent formation of NO (Figure 3). To determine if N-nitrosation proceeded through the formation of peroxynitrite (via the interaction of NO with superoxide), superoxide dismutase (SOD) (100 μg/ml) was added in some experiments. The addition of SOD did not significantly alter the level of nitrosating activity.

DISCUSSION

It has been known for several decades that chronic inflammation is associated with an increased incidence of malignant transformation of epithelial cells (18). Although the mechanisms by which inflammation promotes neoplasia are not known, it has been suggested that inflammatory cells initiate and promote tumor formation via their production of endogenous carcinogenic compounds (18). Although much of this work has focused on oxygen-derived free radicals, more recent studies suggest that certain

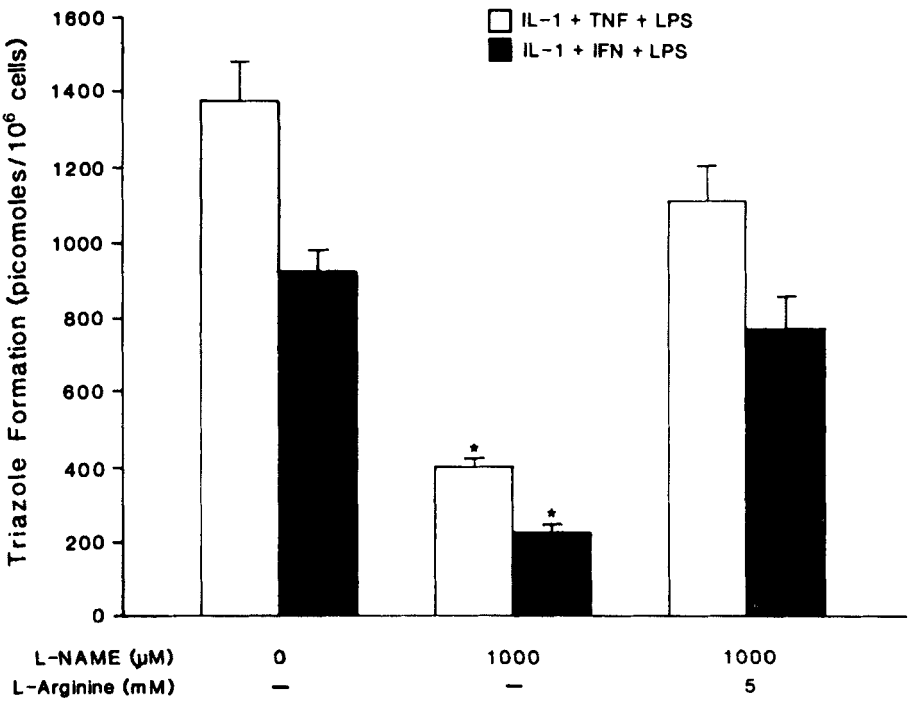

FIGURE 3 Inhibition by N^G-nitro-L-arginine methyl ester (L-NAME) of N-nitrosating activity of confluent monolayers of rat pleural mesothelial cells stimulated for 72 hours with IL-1 (100 ng/ml) + TNF (50 ng/ml) + LPS (50 ug/ml) – □ or IL-1 (100 ng/ml) + IFN (500 units/ml) + LPS (50 ug/ml) – ■. Inhibition of N-nitrosating activity by L-NAME (1 mmol/L) was reversed by the addition of L-arginine (5 mmol/L). Results are expressed as mean±standard deviation (n = 4). Three separate experiments yielded similar results. *P<0.05, samples with L-NAME added vs. samples without L-NAME.

inflammatory cells such as neutrophils, monocytes, and macrophages may promote mutagenesis and possibly carcinogenesis via the formation of NO and its derivatives (7,8). NO is known to rapidly and spontaneously interact with molecular O_2 to yield a variety of nitrogen oxides including NO_3^- and NO_2^-, as well as potent N-nitrosating agents such as NO_2, N_2O_3, and N_2O_4 (7). These nitrosating products will rapidly N-nitrosate primary and secondary amines to yield nitrosamine intermediates. Nitrosamine formation may promote mutagenesis and carcinogenesis by two possible mechanisms. One mechanism dictates that secondary aliphatic nitrosamines are activated into mutagenic and carcinogenic species via the action of the cytochrome P-450 system (19). Cytochrome P-450-mediated metabolism of nitrosamines produces electrophilic agents that will rapidly alkylate a variety of different nucleophilic sites in cellular components, including protein, DNA, and RNA. NO-derived nitrosating agents may also be mutagenic by virtue of their ability to produce base substitution via nitrosative deamination of DNA bases (20,21).

In this study we have demonstrated that PMC stimulated with specific combinations of cytokines and LPS have the ability to N-nitrosate a model primary aromatic amine

to yield its triazole derivative. Furthermore, N-nitrosating activity was inhibited by L-NAME and recovered by the addition of L-arginine, suggesting that the nitrosating agent(s) were derived ultimately from NO. Additionally, N-nitrosation apparently does not proceed through peroxynitrite, since the addition of SOD did not decrease the level of nitrosating activity. The amount of triazole produced correlated with the amount of nitrite plus nitrate produced in a consistent manner. The ratio of nitrosamine formed to total nitrite plus nitrate produced was approximately 0.01, suggesting that (a) only a small fraction (1%) of the nitrogen oxides produced by PMC are nitrosating species and/or (b) because of competing reactions only a small fraction of the nitrosating agent is available for the reaction. The latter possibility appears likely since omission of FBS enhances quite dramatically the nitrosating activity of the cells (data not shown). The results of the present study demonstrate that PMC *in vitro* have the capacity to produce potentially carcinogenic substances in response to proinflammatory cytokines and LPS in a manner that is dependent on the formation of NO.

Although transformation of pleural cells *in vivo* into mesothelioma cells occurs after asbestos-induced injury and inflammation, the mechanism of this alteration remains unclear. Asbestos has been shown to interact with metaphase chromosomes of PMC *in vitro*, resulting in DNA mutations (22). In addition to this direct effect the close proximity of PMC and mononuclear cells during chronic asbestos-induced pleural inflammation suggests that leukocyte-derived products, such as cytokines and oxidants, may also play a role in the transformation process (2–5). A proposed mechanism for the development of mesotheliomas is that PMC DNA damage results from the generation of oxygen free radicals by macrophages that have taken up fibers with subsequent clonal expansion by mitogenic factors elaborated by macrophages or PMC themselves (23). Our results suggest that proinflammatory cytokines may contribute to PMC transformation in an additional manner. It could be postulated that pleural damage and inflammation induced by asbestos fibers may cause an excessive release of proinflammatory cytokines which could directly stimulate the PMC to produce N-nitrosating agents via the formation of NO. It is also possible that asbestos may directly stimulate the PMC to produce NO, as has been recently demonstrated in A549 epithelial cells (24), and hence N-nitrosating agents. The formation of these N-nitrosating agents on a chronic basis could conceivably contribute to the transformation of pleural cells into mesothelioma cells via the mechanisms discussed above.

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