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NITRIC OXIDE PRODUCTION FROM MACROPHAGES IS REGULATED BY ARACHIDONIC ACID METABOLITES

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SUMMARY: In activated macrophages the inducible form of the enzyme nitric oxide (NO) synthase generates high amounts of the toxic mediator NO. After 20 h of treatment with LPS rat peritoneal macrophages release 12-16 nmol $NO_2^-/10^5$ cells which is detectable in the culture supernatant by the Griess reaction as a measure of NO formation. The addition of aminoguanidine (1 mM), a preferential inhibitor of the inducible NO-synthase, completely abolished NO_2^- accumulation. Incubation with indomethacin or acetyl-salicylic acid, preferential inhibitors of the cyclooxygenase pathway of the arachidonic acid metabolism, did not influence NO_2^- levels. Nordihydro-guaiaretic acid (50 μM), a preferential inhibitor of the lipoxygenase pathway, caused strong reduction of NO_2^- accumulation to 1.9 ± 0.3 nmol/200 μl. Simultaneous inhibition of cyclo- and lipoxygenase by BW755c resulted in an intermediate effect (7.3 ± 1.1 nmol/200 μl NO_2^-). These results show that the induction of NO production in activated macrophages is regulated by products of the lipoxygenase-pathway of the arachidonic acid metabolism.

Among a variety of mediators released by activated macrophages (1), nitric oxide (NO) was identified as a potent molecule, which may exert regulatory or cytotoxic effects depending on the concentration acting on the target cell (2, 3). The inducible form of the arginine-dependent enzyme NO-synthase generates high, toxic amounts of NO which enables activated macrophages to destroy tumor cells, parasites, intracellular bacteria (3) and even normal tissue in situations of autoimmune reactivity (4).

Earlier studies have shown that functions of activated macrophages such as tumor cell killing, the release of tumor necrosis factor α (TNF α) and the generation of oxygen radicals are regulated by products of the arachidonic acid metabolism (5-8). In cultured rat peritoneal macrophages PGE_2 was found to exert a strong modulatory effect on the formation of TNF α (6). Deficient PGE_2 production was observed in conjunction with hypersecretion of TNF α (7). Furthermore inhibition of the arachidonic acid cascade was found to be associated with a reduced production of cytotoxic reactive oxygen species (8). Recent observations in a murine macrophage cell line indicate that exogenous PGE_2 may

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Abbreviations: NO, nitric oxide; LPS, lipopolysaccharide.

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suppress the formation of NO (9). On the other hand, when liver macrophages were activated with LPS, NO release was enhanced in the presence of exogenous PGE_2 (10). The present study was designed to investigate the role of endogenous arachidonic acid metabolism in the regulation of NO release from activated macrophages.

MATERIALS AND METHODS

Preparation of macrophages. Macrophages were isolated from 60 - 90 d old Wistar rats provided from our own breeding colony. The animals received 0.5 ml of a heat-inactivated suspension of *Corynebacterium parvum* i.p. (Wellcome, Burgwedel, FRG) and after 5 days the peritoneal cells were harvested by flushing the peritoneal cavity with Dulbecco's PBS at 4 °C (GIBCO Europe, Heidelberg, FRG). The cell suspension was incubated at 37 °C / 5 % CO_2) in plastic petri dishes coated with fetal calf serum (FCS, low in endotoxin, GIBCO). After 1h the non-adherent cells were flushed off and the remaining adherent macrophage population was detached by incubation (4 °C, 10 min) in Ca^{2+} -/Mg^{2+} -free Hank's balanced salt solution. For the subsequent experiments the macrophages were resuspended in RPMI 1640 (GIBCO) supplemented with ampicillin 25 mg/l, penicillin 120 mg/l, streptomycin 270 mg/l (Serva GmbH, Heidelberg, FRG), sodium pyruvate 1 mM, L-glutamine 2 mM, non-essential amino acids (100x) 10 ml/l (GIBCO), $NaHCO_3$ 2g/l, HEPES 2.38 g/l (Serva) and 10 % FCS.

Inhibitors of arachidonic acid metabolism. The following drugs were used as inhibitors of the arachidonic acid metabolism in the cultivated macrophages: 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid (indomethacin, Sigma, Deisenhofen, FRG), acetylsalicylic acid (ASA, Sigma), nordihydroguaiaretic acid (NDGA, Sigma), and 3-amino-1(3-trifluoromethyl-phenyl)-2-pyrazoline hydrochloride (BW755c, Wellcome). Aminoguanidin (Sigma), a preferential inhibitor of inducible NO-synthase, was used as control substance. The drugs were used only in nontoxic concentration ranges. Macrophage viability was >95% in all the samples as assessed by Trypan blue staining.

Nitrite determination. NO release from macrophages was assessed by determination of the NO_2^--concentration in the culture supernatant using the Griess reaction (11). Macrophages were seeded at 1 x 10^5 cells per well (200 µl) of 96 well flat bottom microtiter plates and incubated at 37 °C / 5 % CO_2. Inhibitors of the arachidonic acid metabolism were added at increasing concentrations and after 3 h of preincubation a set of samples was treated with 1 ng/ml lipopolysaccharide (LPS; E.coli 026:B6, Sigma). After 20 h 60 µl of the supernatant were removed, mixed with 80 µl sulfanilamide (1 % in 4 N HCl, Sigma) and 10 µl of concentrated HCl were added. After 10 min 60 µl of N-(1-naphthyl)-ethylenediamine (1 % in methanol, Sigma) were added and the optical density of the resulting solution was determined photometrically (540 nm). The NO_2^- concentrations in the macrophage supernatants were calculated from a standard curve obtained with $NaNO_2$.

Statistical analysis. For statistical evaluation of the data, the Student's t-test was used.

RESULTS AND DISCUSSION

Activated rat peritoneal macrophages (1 x 10^5 cells/200 µl) were incubated for 20 h and the concentration of the accumulated NO_2^- was determined in the culture supernatant as a measure of NO release. As shown in Fig. 1, in the absence of an inhibitory agent LPS treated macrophages released 12-16 nmol / 200 µl NO_2^- which corresponds to our previous findings (12). In order to prove that the accumulation of NO_2^- is due to the activity of the

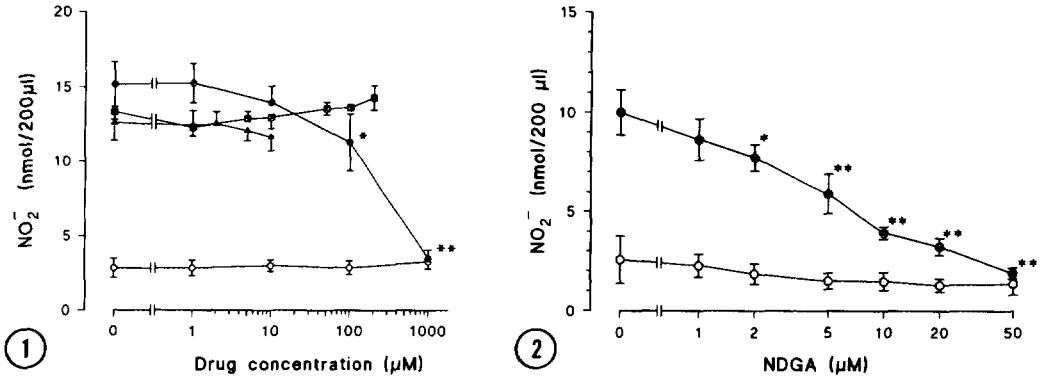

Fig. 1. Effects of NO synthase- and cyclooxygenase inhibitors on NO_2^- accumulation in cultures of activated macrophage cultures. Macrophages (1 x 10^5 cells/200 µl) were treated with 1 ng/ml LPS and incubated in the presence of increasing concentrations of the cyclooxygenase inhibitors indomethacin (▲) or acetyl-salicylic acid (■) or the NO synthase inhibitor aminoguanidine (●) as control. After 20 h the accumulated NO_2^- was determined in the supernatant by the Griess reaction (see Materials and Methods) as a measure of NO production. In the absence of LPS the macrophage cultures released 2.8±0.7 nmol/200 µl NO_2^- which remained unchanged in the presence of the inhibitory substances (a representative control curve for aminoguanidine is shown (○). Data show mean ± SD from three separate experiments performed in triplicate. * $p < 0.01$, ** $p < 0.001$ compared to the control without drug.

Fig. 2. The lipoxygenase inhibitor NDGA dose dependently inhibits the accumulation of NO_2^- in macrophage cultures. LPS stimulated (●) or unstimulated (○) macrophages were cultivated with increasing doses of NDGA. After 20 h the NO_2^- concentration in the supernatant was determined by the Griess reaction. Data show mean ± SD from three experiments performed in triplicate. * $p < 0.01$, ** $p < 0.001$ compared to the control without NDGA.

inducible NO synthase, aminoguanidine was added, which is known to be a potent inhibitor of this enzyme (13). As shown in Fig. 1 aminoguanidine strongly reduced NO_2^- formation. At a concentration of 1 mM of the substance the accumulation of NO_2^- in the macrophage supernatant was completely abolished ($p<0.001$). In the absence of LPS the macrophages released only low amounts of NO_2^-, which were unaffected by aminoguanidine or by any other substance tested in the following experiments. These results indicate that in our test system the inducible form of the NO synthase is the major source of NO from LPS treated macrophages.

The role of endogenous arachidonic acid metabolites in the regulation of macrophage NO formation was tested by adding inhibitors of the arachidonic acid metabolism to macrophages 3 h prior to LPS.

In a first series of experiments indomethacin and acetyl-salicylic acid were added to the macrophage cultures. Both drugs are known to inhibit the cyclooxygenase pathway of the arachidonic acid metabolism (14, 15). As shown in Fig. 1 both substances did not influence NO_2^- accumulation over wide ranges of concentrations. These observations indicate that while exogenous PGE_2 may suppress or enhance NO production in LPS treated macrophages (9, 10) endogenous PGE_2 does not contribute to the induction of NO synthase following LPS. Both acetyl-salicylic acid and indomethacin are potent inhibitors of PGE_2

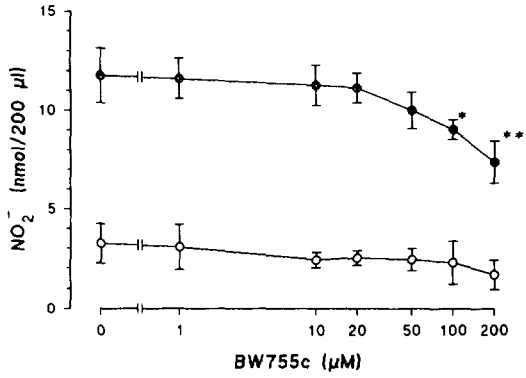

Fig. 3. Reduction of NO_2^- accumulation in macrophage cultures by BW755c, an inhibitor of cyclo- and lipoxygenase. LPS stimulated (●) or unstimulated (○) macrophages were cultivated in the presence of rising concentrations of BW755c and after 20 h the amount of NO_2^- in the culture supernatant was determined. Data show mean ± SD from three separate experiments performed in triplicate. * $p < 0.01$, ** $p < 0.001$ compared to the control without BW755c.

formation, and even maximal nontoxic doses did not significantly reduce NO_2^- accumulation over 20 h of macrophage culture.

In the next experiment the lipoxygenase pathway was investigated for its involvement in NO release from activated macrophages. As shown in Fig. 2 activation of macrophages in the presence of NDGA, a preferential inhibitor of lipoxygenase (14), resulted in a strong and dose dependent reduction of NO_2^- formation from 10.0 ± 1.1 nmol/200 µl to 1.9 ± 0.3 nmol/200 µl ($p < 0.0001$).

Finally BW755c was tested which acts as inhibitor of both the lipoxygenase and cyclooxygenase pathways (16,17). Addition of the drug had an intermediate effect on LPS inducible NO_2^- accumulation in the macrophage cultures (Fig. 3). A dose of 200 µM of BW755c significantly reduced the NO_2^- concentration from 11.8 ± 1.4 nmol/200 µl to 7.3 ± 1.1 nmol/200 µl ($p < 0.001$).

The latter results indicate that products of the lipoxygenase pathway of the arachidonic acid metabolism are involved in the induction of NO release from LPS treated macrophages. Such conclusion is supported by our earlier observation that NDGA suppresses the cytotoxic actions of macrophages against pancreatic islet cells in a cocultivation assay, which serves as a model of inflammatory destruction of normal cells (18). The lytic action could also be suppressed by N-methyl-arginine, an inhibitor of constitutive and inducible NO synthases (19). These results also correspond to the observations by Ryoyama (20) who investigated NO mediated tumor cell damage by mouse peritoneal macrophages. In this experimental system NDGA inhibited macrophage antitumor activity, whereas indomethacin was uneffective.

Taken together with the results of our present study it is concluded that products of the lipoxygenase pathway of the arachidonic acid metabolism are important modulators of NO release from activated macrophages and therefore may contribute to the regulation of NO mediated cytotoxicity of these effector cells.

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