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PLANT NITRATE REDUCTASE GENE FRAGMENTS ENHANCE NITRITE PRODUCTION IN ACTIVATED MURINE MACROPHAGE CELL LINES

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Nitrate reductase (NR) gene fragments (1.1 kb and 800 bp) from the barley plant were incorporated into pSV_2neo and transfected by electroporation into a variety of cell lines of different functionality. Only transfected murine macrophage cell lines demonstrated appreciably enhanced NO_2^- production (i.e., NR activity) both in the presence and absence of exogenous nitrate (NO_3^-). Addition of NO_3^- caused the greatest increase in NO_2^- production when macrophages were primed with interferon-gamma (INF-γ) and lipopolysaccharide (LPS). Transfection of RAW 264.7 murine macrophages led to isolation of several novel neomycin-resistant subpopulations designated $NR10_1$, $NR10_2$ (both containing the 1.1 kb NR fragment) and $NR800_5$ (containing the 800 bp NR fragment). Similarly transfected nonleukocytic and leukocytic stem cell lines showed no significant NO_2^- production. Outside of the macrophage cell lines, only the murine T cell line EL-4 showed evidence of mild nitrite production enhancement. The mechanism of enhanced NO_2^- formation in NR transfected murine macrophages is unknown. However, study of these novel cells may lead to greater understanding of the expression of a plant NR in mammalian cells and highly controlled production of a cytotoxic molecule (NO_2^-) in macrophages.

In the murine immune system, L-arginine-dependent production of nitric oxide (NO) and its spontaneous oxidation products (NO_2^- and NO_3^-) by phagocytes appears to require stimulation from specific ligands such as lipopolysaccharide (LPS) or interferon (IFN; 1-7). Oxidized nitrogen species are generated by nitric oxide synthase (NOS) isozymes as nonoxidative microbicidal, tumoricidal, or inhibitory agents (1-7) and possibly as cytokines (1, 2).

Due to the spontaneous oxidation and short half-life of NO, it is difficult to separate the cellular effects of NO from potential effects of NO_2^-, NO_3^- or their derivatives (e.g., S-nitrosothiols, peroxynitrites, nitrsoamines, etc., 2, 5). Thus, it would be desirable to circumvent the NOS pathway and produce high concentrations of NO_2^- or NO_3^-. To study the feasibility of circumventing NOS, fragments of the barley plant nitrate reductase (NR) were transfected into a wide variety of cell lines to determine if NO_2^- might be produced independently of NO. NR

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cDNA fragments were used because the entire gene was unavailable. Data presented here, however, suggest that the NR fragments were expressed and were somehow dependent on NOS or another redox system in activated macrophages to complete the reduction of NO_3^- to NO_2^-.

Materials and Methods

Materials

Griess reagents (0.8 % sulfanilic acid and 0.5% N,N-dimethyl-alpha-naphthylamine, both in 5N acetic acid) were obtained from Baxter Medical Corp. (Sacramento, CA.) All cell lines were obtained from American Type Culture Collection, Rockville, MD. Murine INF-γ and LPS from *E. coli* serotype 055:B5 were obtained from Sigma Chem. Co. (St. Louis, MO) and stock solutions of each were prepared in phosphate buffered saline (PBS; pH 7.4). The *Limulus* amoebocyte lysate assay kit was also obtained from Sigma. Geneticin (neomycin) was obtained from GIBCO (Grand Island, NY).

Preparation of Plasmids

Plasmid pBcNR/10 containing a 1.1 kb fragment of the barley nitrate reductase gene (a gift from Dr. Howard Goodman, Massachusetts General Hospital; 8) was extracted from *E. coli* (HB101) grown in M9 minimal medium, amplified with chloramphenicol and extracted by banding twice in cesium chloride gradient. The resulting DNA was extracted with phenol/chloroform and ethanol precipitated. Concentration of DNA was determined spectrophotometrically at 260 nm. The 1.1 kb fragment was derived from a Pst I digest of pBcNR/10. The 800 bp fragment resulted from a Sal I digest of the 1.1 kb fragment. EcoR1 linkers were added to both fragments for incorporation into pSV_2neo.

Transfection

Fifty million cells of each cell line examined were washed twice in cold PBS and suspended in 1 ml of cold RPMI-1640 without serum, phenol red, or antibiotics (9, 10). Approximately 19 to 30 μg of pSV_2neo plasmid DNA containing the barley NR gene fragments (8) were added to the cell suspensions and left on ice for 10 minutes (9, 10). Electroporation was accomplished using the BioRad GenePulser apparatus operated at 450 V and 25 μFaraday (X 5 pulses). $NR10_1$ and $NR10_2$ were derived by transfection of RAW 264.7 cells with pSV_2neo containing the 1.1 kb NR insert. $NR800_5$ was derived by transfection of RAW 264.7 cells with pSV_2neo containing the 800 bp NR insert.

Cell Culture, INF-γ/LPS Activation and Nitrite (Griess) Assay

Following electroporation, cells were placed on ice for an additional 10 minutes and transferred to 25 ml of RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS) except for the B-16 melanoma cells (cultured in Ham's F10 plus 15% non-heat inactivated horse serum and 2.5% non-heat inactivated FBS) and allowed to stabilize in culture at 37° C and 5% CO_2 for 72 hours. Dead cells were decanted and fresh medium without phenol red was added. Adherent cells were allowed to grow to confluency and were subcultured twice prior to use in experiments. Selection of the $NR10_1$, $NR10_2$ and $N800_5$ macrophage clones was achieved by addition of 500 μg/ml geneticin (neomycin). Culture media were made from ultra-pure deionized water kept free of bacterial growth. Water, media and FBS repeatedly tested negative for LPS contamination. Cells were plated in microtitre (96-well) plates at 10^4 per well in 100 μl aliquots. Macrophages were activated by incubation in 10 U/ml murine INF-γ and 5 ng/ml LPS for 48 hrs. Subsequently medium was removed and replaced with fresh medium or medium containing 39.5 mM KNO_3 for 24 hrs. Cells appeared to tolerate the presence of NO_3^- well and were observed to attach normally in medium containing KNO_3. One hundred μl of each Griess reagent were added to each well and plates were incubated at 37°C for 15 min. Results were quantified with a BioRad Model 450 microplate reader (655 nm emission filter). A standard curve was determined and used to convert absorbance values into μM NO_2^- concentrations. Griess reaction products were removed from the wells. Wells were washed twice with 300 μl of PBS and allowed to air dry. Thereafter, wells were fixed briefly in 100 μl of methanol and Wright stained. Relative adherent cell density was assessed from the O.D. at 405 nm of each well by use of the microplate reader.

Results

Fig. 1 shows NO_2^- production levels of unstimulated and INF-γ/LPS stimulated $NR800_5$ macrophages in the presence and absence of exogenous KNO_3 and illustrates the dependence of

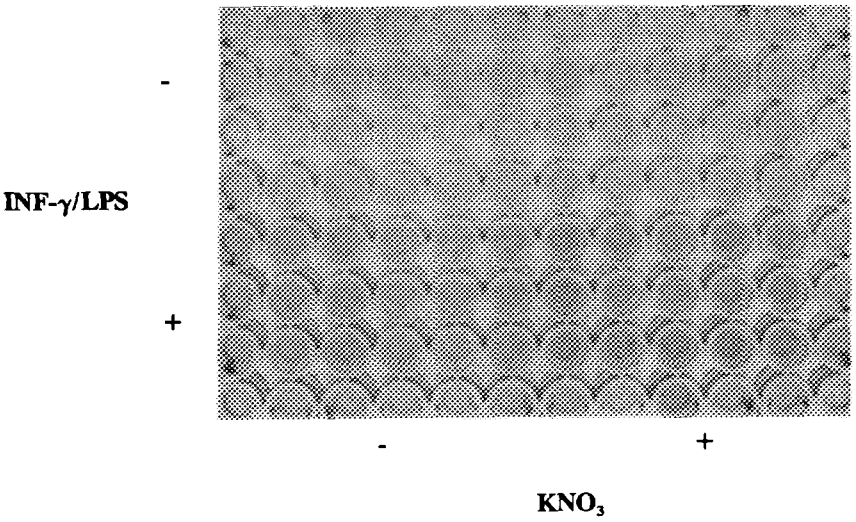

Fig. 1. Appearance of a typical Griess assay performed in a microtitre plate for the NR800$_5$ cells. The upper left quadrant was neither stimulated nor cultured with KNO_3. The upper right quadrant was not stimulated, but was cultured with KNO_3. The lower left quadrant was stimulated with INF-γ and LPS (see Materials and Methods), but not cultured with KNO_3. The lower right quadrant was INF-γ and LPS stimulated and was cultured with KNO_3 and shows the greatest NO_2^- production.

enhanced NO_2^- production on INF-γ/LPS induction. Among unstimulated cells, there appeared to be little NO_2^- production dependence on the presence of KNO_3. The minor NO_2^- levels produced by unstimulated cells may arise from low level constitutive NOS (cNOS) activity which has been recently demonstrated in RAW 264.7 cells (11).

Fig. 2 summarizes NO_2^- assay results for transfected and control (nontransfected) RAW 264.7 cells at two days after INF-γ/LPS priming, removal of stimulants, and one day after addition of KNO_3. Transfectants (NR10$_1$ and NR10$_2$ cells) pretreated with INF-γ and LPS followed by addition of KNO_3 demonstrated the greatest NO_2^- production. Data in fig. 2 were normalized for cell density by dividing μM NO_2^- concentrations by the absorbance at 405 nm following a wash step and staining with Wright Stain.

Enhanced NO_2^- production has been demonstrated primarily in murine macrophage cell lines (Table I). However, 1.1 kb NR transfected EL-4 murine thymoma cells also appeared to demonstrate a very slight enhancement of NO_2^- production (data not shown). NR transfected leukocytic stem cells such as HL-60, U937, and K562 showed no detectable enhancement of NO_2^- production. Similarly, transfected cell lines of nonleukocytic origin (i.e., B16 melanoma cells, HeLa, or 3T3 fibroblasts) did not show evidence of augmented NO_2^- production.

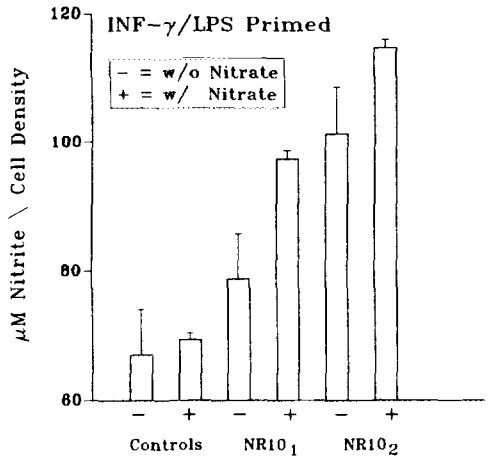

Fig. 2. Ratio of absorbance of the Griess reaction product (NO_2^- assay) at 655 nm to the absorbance of Wright stained adherent cells (cell density) at 405 nm. This ratio measures NO_2^- production on a per cell basis. The figure compares this ratio for INF-γ/LPS stimulated controls (nontransfected RAW 264.7 cells), $NR10_1$, and $NR10_2$ transfectants in the presence (+) and absence (-) of 39.5 mM KNO_3. Data represent the means and standard deviations of 32 measurements taken from 2 separate microtiter plate experiments.

Discussion

This work demonstrates that development of transfected cell lines which express high levels of NO_2^- from reduction of NO_3^- is possible. The mechanism of NO_2^- production enhancement is unknown. However, data presented here imply that the truncated NR fragments may be dependent on another redox system peculiar to some leukocytes (especially activated murine macrophages) to complete NO_3^- reduction to NO_2^-. The evidence for this hypothesis is

TABLE I

Qualitative Results of Transfection with the 1.1 kb Barley Nitrate Reductase Gene Fragment

Cell Line	Type	Reaction*
RAW 264.7	Murine Macrophage	+
P388D1	Murine Macrophage	+
J774A.1	Murine Macrophage	+
EL-4	Murine Thymoma	+
HL-60	Human Promyelocyte	-
U937	Human Monoblast	-
K562	Human Erythroblast	-
B16	Murine Melanoma	-
HeLa	Human Cervical Carcinoma	-
NIH 3T3	Murine Fibroblast	-

* Plus indicates that a given cell line showed enhanced nitrite production. Negative cell lines were transfected and assayed at least twice.

several fold. Firstly, the enhanced NO_2^- production is only observed in leukocytic (primarily macrophage) cell lines (Table I). Secondly, NO_2^- production of NR transfected RAW 264.7 cells is enhanced over that of nontransfected controls even in absence of added NO_3^- (Fig. 2). The fact that exogenous KNO_3 is not necessary to the process suggests an endogenous source of NO_3^- such as iNOS (but not due to LPS contamination; ruled out by *Limulus* amoebocyte lysate testing) or a constitutive NOS (cNOS) activity (recently reported in RAW 264.7 cells; 11). Thirdly, addition of NO_3^- further enhances NO_2^- production among INF-γ/LPS stimulated transfectants. Murine leukocytes are known to possess at least iNOS, and possibly cNOS, activity while the other cell types examined here are not known to have appreciable NOS activity. If the enhanced NO_2^- production were due to a native NR activity not previously described in macrophages, then control cells, with or without activation, would have shown significantly increased NO_2^- production upon culturing with KNO_3. Instead, the data suggest NR activity only in transfected macrophages which is somehow dependent on INF-γ/LPS stimulation (and possibly therefore dependent on NOS). It should be noted that while cytokine or other stimulation is not always necessary to produce the enhanced NO_2^- production, pretreatment with INF-γ and LPS makes the NO_2^- enhancement phenomenon consistently visible.

Neomycin selection of transfected macrophage cell lines indicates that the NR fragments inserted in pSV_2neo are present in transfectants and are probably expressed since the neomycin resistance gene is expressed. It is noteworthy that the NO_2^- production of the $NR10_1$, $NR10_2$ and $NR800_5$ cells differed somewhat. This difference may reflect insertion into different sites in the macrophage genome. Insertion at different genomic sites may also account for the variable growth kinetics observed (data not shown) among the transfectants.

It is generally remarkable that the putatively expressed NR fragments are capable of nitrate reduction being that they represent about a third or less of the entire (3 kb) barley NR gene (8, 12). Thus, the truncated proteins coded for by these fragments must contain the critical functional domains necessary to reduce NO_3^- at least upon synergizing with extant redox systems.

Regardless of the mechanism of enhanced NO_2^- production, it is clear that NO_2^- production was augmented by transfection with the barley NR DNA fragment. Enhanced cellular NO_2^- production has potential applications to metabolic studies of nitrogen oxide species. Additionally, such methodology could find medical applications in the augmented killing of tumors, obstinate microorganisms, or viruses *in vivo* by transfected macrophages whose NO_2^- production could be tightly regulated by control of exogenous NO_3^- levels.

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