BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

SELECTIVE TARGETING OF NITRIC OXIDE SYNTHASE INHIBITORS TO SYSTEM y^+ IN ACTIVATED MACROPHAGES

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SUMMARY: Amino acid transport systems mediating uptake of nitric oxide (NO) synthase inhibitors were characterized in the murine macrophage cell line J774. Treatment of J774 cells with bacterial endotoxin (LPS, 1 μg ml^{-1}, 24 h) selectively increased the transport capacity for N^G-monomethyl-L-[^{14}C]arginine (L-NMMA), whereas transport of N^G-nitro-L-[^{3}H]arginine (L-NNA) was unaffected. Inhibition studies established that the cationic transport system y^+ mediates uptake of L-arginine, L-NMMA and N^G-iminoethyl-L-ornithine (L-NIO). A neutral transporter, with low substrate specificity and insensitive to LPS, mediates uptake of L-citrulline, L-NNA and its methyl ester L-NAME. We conclude that enhanced expression of the y^+ transporter in LPS-stimulated macrophages (1) may facilitate the targeting of selective inhibitors of inducible NO synthase to activated cells generating NO in endotoxin shock.

Activation of murine macrophage J774 cells with lipopolysaccharide (LPS) results in a cycloheximide-dependent induction of NO synthase and arginine transport via system y^+ (1-3). In activated macrophages generation of NO by inducible NO synthase (iNOS) is critically dependent on extracellular arginine (2,4,5), despite reports that activated cells contain high intracellular arginine levels (1,6).

N^G-derivatized analogues of L-arginine are potent inhibitors of iNOS, and their rank order of potency has been attributed to different NOS isoforms and/or their differential rates of cellular uptake (7,8). In this context, we reported previously that N^G-monomethyl-L-arginine (L-NMMA) and N^G-iminoethyl-L-ornithine (L-NIO), but not N^G-nitro-L-arginine (L-NNA) nor its methyl ester L-NAME inhibit transport of arginine into cultured porcine aortic endothelial cells via system y^+ (9). Recent studies of radiolabelled L-NMMA and arginine uptake in porcine aortic endothelial cells have confirmed these findings (10).

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Our recent finding that the y^+ amino acid transporter is induced in LPS-stimulated macrophages (1-3) led us to hypothesize that enhanced expression of system y^+ could be exploited in targeting L-NMMA, an inhibitor currently under clinical trial, to activated vascular cells in endotoxin shock. Our data confirm this hypothesis and establish that transport of L-NMMA and L-NIO are mediated by system y^+, whereas a neutral transporter insensitive to endotoxin mediates uptake of L-NNA, L-NAME and L-canavanine.

MATERIALS AND METHODS

Materials

All reagents for cell culture except foetal calf serum (GlobePharm Laboratories Ltd) were from Gibco. *Escherichia coli* lipopolysaccharide (LPS, serotype 055:B5) was obtained from Difco, Michigan, USA, and all other chemicals were obtained from Sigma. Unlabelled L-NMMA and L-NIO were gifts from Dr Salvador Moncada FRS, Wellcome Research Lab., Kent. L-[2,3-^{3}H]arginine (53 Ci/mmol), L-[*carbamoyl*-^{14}C]citrulline (54.3 mCi/mmol), N^G-nitro-L-[2,3,4,5-^{3}H]arginine (61 Ci/mmol) and N^G-methyl-L-[5-^{14}C]arginine monohydrochloride (56 mCi/mmol) were obtained from Amersham International plc.

Cell culture

The murine monocyte/macrophage cell line J774 was obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire). J774 cells were maintained in continuous culture in Dulbecco's modified Eagles medium (DMEM) containing 0.4 mM L-arginine and supplemented with 4 mM glutamine, penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and 10% foetal calf serum. Cells were harvested by gentle scraping and passaged every 3-6 days by dilution of a suspension of the cells 1:10 in fresh medium.

Measurement of unidirectional amino acid transport and nitrite production

J774 cells were plated in 96-well microtiter plates (10^5 cells per well) and allowed to adhere for 2 h. Medium was then replaced with either fresh DMEM or with DMEM containing LPS (1 $\mu g\ ml^{-1}$) for 24 h. Cells were rinsed twice with a modified Hepes-buffered Krebs solution (mM: NaCl, 131; KCl, 5.5; $MgCl_2$, 1; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1; D-glucose, 5.5; Hepes, 20; pH 7.4) maintained at 37^{o}C (1-3). Amino acid uptake was initiated by adding Hepes-buffered Krebs (50 µl per well; 37^{o}C) containing either L-[^{14}C]citrulline, L-[^{3}H]arginine, L-[^{3}H]NNA or L-[^{14}C]NMMA (1 $\mu Ci\ ml^{-1}$) to the monolayers. Incubations were terminated after 30 s by placing the plates on melting ice and rinsing cells three times with 200 µl ice-cold Dulbecco's phosphate-buffered saline containing 10 mM unlabelled substrate to remove extracellular radiolabelled amino acids. In some experiments an extracellular reference tracer either D-[^{14}C]mannitol or D-[^{3}H]mannitol was included in the incubation medium. The efficiency of the washing procedure was confirmed by a recovery in the cell lysate of < 0.01% of D-mannitol applied (data not shown).

Cell protein was determined using the BioRad reagent and radioactivity in formic acid digests of the cells was determined by liquid scintillation counting. Uptake was expressed in units of pmol $\mu g\ protein^{-1}\ min^{-1}$. Nitric oxide production was monitored by measuring nitrite accumulation in the culture medium using the Griess reaction (1-3,11).

All values are mean ± S.E.M. of n replicate measurements in at least 3 separate experiments. Analysis was by Student's t-test and $P < 0.05$ was considered as statistically significant.

RESULTS

In a previous study we established that entry of arginine into J774 cells is mediated selectively via the y^+ transporter, whereas citrulline is taken up via a neutral carrier with broad substrate specificity (3). These two systems appear to be the major transporters mediating amino acid entry into J774 cells. Using L-[^{3}H]arginine and L-[^{14}C]citrulline as transport probes, we have now examined the inhibitory effects of various arginine analogues, known to inhibit NOS (see review 12), and identified the transport systems mediating uptake of these compounds by J774 cells.

In agreement with our earlier findings (1), incubation of J774 cells with LPS (1 μg ml^{-1}, 24 h) resulted in an induction of arginine transport and iNOS. Nitrite production increased from 0.1 ± 0.02 to 5.3 ± 0.2 nmol μg protein^{-1} 24 h^{-1}. Under these conditions, uptake of arginine was markedly inhibited by L-NMMA (K_i = 0.58 mM) and L-NIO (K_i = 0.58 mM) and to a much lesser extent by L-NNA and L-NAME (Fig. 1a). Unlike arginine, transport of citrulline was unaffected by LPS and inhibited by L-NNA (K_i = 0.34 mM), L-NAME (K_i = 0.53 mM) but not by L-NMMA or L-NIO (Fig. 1B). By comparison canavanine, an inhibitor of NO synthesis in phagocytic cells (4), only marginally reduced arginine transport but inhibited citrulline transport by ~50% in control and LPS-activated cells.

Experiments using radiolabelled L-NNA and L-NMMA confirmed that these compounds are transported into J774 cells by saturable mechanisms. As shown in Fig. 2A, L-[^{3}H]NNA transport was saturable (K_t = 0.32 ± 0.08 mM and V_{max} = 89.2 ± 5.1 pmol mg protein^{-1} min^{-1}) and not altered in LPS-activated cells generating NO. In contrast, activation of cells with LPS significantly increased the V_{max} for L-[^{14}C]NMMA transport (control: K_t = 0.03 ± 0.01 mM, V_{max} = 2.8 ± 0.32 pmol mg protein^{-1} min^{-1}; LPS: K_t = 0.03 ± 0.002 mM, V_{max} = 6.3 ± 0.11 pmoles μg protein^{-1} min^{-1})(Fig. 2B). A non-saturable component for NMMA transport was detectable at extracellular NMMA concentrations above 0.5 mM (data not shown).

In kinetic cross-inhibition studies of L-[^{3}H]NNA transport, L-NAME (K_i = 0.39 mM) and citrulline (K_i = 0.36 mM) caused a concentration dependent inhibition, whereas L-NMMA, L-NIO and arginine were poor inhibitors (Fig. 3A). In contrast, L-[^{14}C]NMMA transport was inhibited significantly by L-NIO (K_i = 0.49 mM), arginine (K_i = 0.34 mM) and to a lesser extent by citrulline (-38.5 ± 5.7 % at 5 mM) but not by L-NNA or L-NAME. The partial inhibition of L-[^{14}C]NMMA transport by citrulline is reminiscent of our earlier studies of arginine transport in J774 cells, where citrulline could only inhibit arginine transport in the presence of extracellular Na^+ (3). Such an inhibition of system y^+ by neutral amino acids in the presence of Na^+ is

characteristic property of this cationic transporter (13).

DISCUSSION

In a previous study we established that J774 cells transport citrulline and arginine via a neutral amino acid carrier and a cationic amino acid system y^+, respectively (3). Moreover, we showed that metabolism of citrulline to arginine in activated J774 macrophages could only sustain ~20 % of the maximal rate achieved with arginine, despite comparable or greater transport rates for citrulline.

We have now extended these studies and found that the potent inhibitors of iNOS, L-NMMA and L-NIO, are transported into J774 cells by system y^+. This Na^+-independent, high-affinity transporter mediates uptake of cationic amino acids in other cell types (13). We found that three other inhibitors of iNOS, L-NNA, L-NAME and L-canavanine, were very poor substrates for system y^+, entered J774 cells by a neutral transporter and were subject to marked inhibition by competitor neutral amino acids. This latter non-selective carrier resembles that identified in freshly isolated murine peritoneal macrophages (14). Although a low-affinity cationic amino acid transporter (MCAT-2) has recently been identified in murine hepatocytes and the macrophage cell line RAW264.7, only 5-10% of the MCAT-2 transporter would be occupied by substrate at plasma amino acid concentrations (15,16). As the specificity of arginine transport appears to be similar in unstimulated and activated J774 cells (1-3) and peritoneal macrophages (14), it is likely that LPS enhanced the activity of system y^+ in these cell types rather than inducing the MCAT-2 transporter.

Differences in the rank order potency of inhibitors of iNOS in J774 cells have been reported and McCall *et al.* (8) hypothesized that these differences may reflect uptake of NOS inhibitors by different transport systems. Our findings have confirmed this hypothesis and identified system y^+ as a potential target for inhibitors of iNOS. The selectivity of system y^+ for L-NMMA and L-NIO may be accounted for by their positively-charged guanido and amidino groups (pK_a ~ 13) which renders them strongly basic (see review 12). In contrast, L-NNA and L-NAME have a pK_a ~ 0 and would be neutral at physiological pH.

In conclusion, the selectivity of system y^+ for basic amino acids and its induction by LPS (1-3) provides a unique mechanism for accelerating transport of L-NMMA and L-NIO into activated vascular cells. The design of structural analogues of arginine which exploit the y^+ transporter may enhance not only their delivery but also their potency as inhibitors of iNOS in pathological conditions such as endotoxin shock.

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REFERENCES

1. Baydoun, A.R., Bogle, R.G., Pearson, J.D., and Mann, G.E. (1993). Br. J. Pharmacol. **110**, 1401-1406.
2. Bogle, R.G., Baydoun, A.R., Pearson, J.D., Moncada, S., and Mann, G.E. (1992a). Biochem. J. **284**, 15-18.
3. Baydoun, A.R., Bogle, R.G., Pearson, J.D. and Mann, G.E. (1994). Br. J. Pharmacol., **paper in press**.
4. Hibbs, J.B. Jr, Vavrin, Z., and Taintor, R.R. (1987). J. Immunol. **138,** 550-565
5. Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D., and Wishnok, J.S. (1988). Biochemistry **27**, 351-356.
6. Hecker, M., Sessa, W.C., Harris, H.J., Anggard, E.E., and Vane, J.R. (1990). Proc. Natl. Acad. Sci. **87**, 8612-8616.
7. Gross, S.S., Stuehr, D.J., Aisaka, K., Jaffe, E.A., Levi, R., and Griffith, O.W. (1990). Biochem. Biophys. Res. Commun. **170**, 96-103.
8. McCall, T.B., Feelisch, M., Palmer, R.M.J., and Moncada, S. (1991). Br. J. Pharmacol. **102**, 234-238.
9. Bogle, R.G., Moncada, S., Pearson, J.D., and Mann, G.E. (1992b). Br. J. Pharmacol. **105**, 768-770.
10. Schmidt, K., Klatt, P., and Mayer, B. (1993). Mol. Pharmacol. **44**, 615-621.
11. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., and Tannenbaum, S.R. (1982). Anal. Biochem. **126**, 131-138.
12. Knowles, R.G., and Moncada, S. (1994). Biochem. J. **298**, 249-258.
13. White, M.F. (1985). Biochim. Biophys. Acta **822**, 355-374.
14. Sato, H., Watanabe, H., Ishii, T., and Bannai, S. (1987). J Biol Chem **262**, 13015-13019.
15. Closs, E.I., Albritton, L.M., Kim, J.W., and Cunningham, J.M. (1993). J. Biol. Chem. **268**, 7538-7544.
16. Closs, E.I., Lyons, C.R., Mitchell, M., and Cunningham, J.M. (1993). Endothelium **1**, S16.

Figure 1. **Inhibition of arginine and citrulline transport in unstimulated and LPS-activated J774 macrophage cells.** Cells were cultured in DMEM in absence (solid bars) or presence of LPS (1 μg ml^{-1} for 24 h, hatched bars), and transport of 100 μM L-[^{3}H]arginine (A) or L-[^{14}C]citrulline (B) was then measured in cells incubated with a Hepes-buffered Krebs medium containing inhibitors of iNOS (1 mM). Values denote the mean ± SE of 10 separate measurements. * $P < 001$ compared to control.

Figure 2. **Kinetics of L-NMMA and L-NNA transport in unstimulated and LPS-activated J774 macrophage cells**. Cells were cultured in DMEM in the absence (■) or presence (⮝) of LPS (1 μg ml^{-1} for 24 h). Monolayers were washed and the kinetics of L-NNA (A) and L-NMMA (B) transport measured over a 30 s during incubation of cells with medium containing 0.025 - 1 mM unlabelled substrate. Michaelis-Menten rectangular hyperbolas were obtained by a direct fit to the mean influx values, estimated from 16 measurements in 3 separate experiments.

Figure 3. **Specificity of L-NNA and L-NMMA transport in unstimulated J774 macrophage cells**. Inhibition of 100 μM L-[^{3}H]NNA (A) or 100 μM L-[^{14}C]NMMA (B) transport by increasing concentrations (0.025 - 5 mM) of L-NMMA (■), L-NAME (○), L-NNA (●), L-NIO (⩛), L-arginine (□) or L-citrulline (⩚). Unidirectional transport of L-[^{3}H]NNA or L-[^{14}C]NMMA was measured over 30 s. Values denote mean ± SE of 10 separate measurements.