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PARALLEL INDUCTION OF NITRIC OXIDE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN ACTIVATED BONE MARROW DERIVED MACROPHAGES+

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The production of nitric oxide (NO·) and the induction of glucose-6-phosphate dehydrogenase by lipopolysaccharides (LPS) from different sources was studied in bone marrow derived macrophages (BMMϕ). NO· production was found to be linked to the induction of glucose-6-phosphate dehydrogenase, suggesting the possible involvement of this enzyme in the cytotoxic mechanism resulting from the release of NO· by activated macrophages.

Nitric oxide is a simple molecule (gas under atmospheric conditions) synthesized, together with citrulline, from arginine by the enzyme nitric oxide synthase (EC, 1.14.23). Nitric oxide reacts readily with molecular oxygen to yield nitrogen dioxide (NO_2) which ultimately reacts to form NO_2^- and NO_3^- via the hydrolysis of N_2O_3 and N_2O_4 (1).

Nitric oxide synthase has recently been purified from cerebellum (2,3) and from macrophages (Mϕ) (4,5), as well as from other sources, and is a cytochrome P-450 type haemoprotein (6,8) as deduced from its nucleotide sequence (6,7). Nitric oxide is a well-known neurotransmitter, blood pressure regulator, and potent antimicrobial, cytotoxic and inflammatory agent (9-11), as well as a pathogenic factor in autoimmunity (12).

All nitric oxide synthases so far purified are classified in two general categories: a) a constitutive form and b) an inducible form (13). Both forms of purified nitric oxide synthase contain tightly-bound FAD and FMN (5,14). The enzyme also requires NADPH (14,15) and 6(R) tetrahydro-L-biopterin (14). The NADPH requirement suggests that cytoplasmic levels of this coenzyme are crucial to NO· biosynthesis; given that a considerable portion of NADPH is

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Abbreviations: BMMϕ, bone marrow derived macrophages; LPS, bacterial lipopolysaccharides; NO·, nitric oxide.

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generated in the oxidative phase of the pentose pathway (16-18), it would not be unreasonable to assume that one of the enzymes involved in this phase, glucose-6-phosphate dehydrogenase, may be regulated during Mϕ activation, a process associated with substantial NO· production. Glucose-6-phosphate dehydrogenase is the most highly-regulated enzyme in the oxidative phase. Its synthesis is controlled by different nutritional and hormonal conditions (16,19,20), and its activity is controlled (21-24) mainly by the cytosol NADPH/$NADP^+$ ratio.

The purpose of this study was to identify the close relationship between the induction of glucose-6-phosphate dehydrogenase and the production of NO· measured as formed nitrite (NO_2^-). Since 50% to 75% of total NO· appears in the form of nitrite (25).

MATERIAL AND METHODS

BMMϕ from CB17 mice were obtained as described (26). Briefly, cells (10^5/ml) from the femur were suspended in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 30% L929 supernatant as a source of colony-stimulation factor, 5% horse serum, 1 mM sodium pyruvate, 2 mM glutamine, 60 μM β-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were cultured for 10 days in 85 cm^2 Teflon bags (Biofolie 25, Heraeus, Hanau, F.R.G.) in 10% CO_2 at 37°C before use. At this time more than 95 % of the cells are BMMϕ. At this stage, cells were washed twice with sterile phosphate-buffered saline without Ca^{2+} or Mg^{2+}, pH 7.4 (PBS) and recultured in DMEM without L929 supernatant at 10^6 cells/ml. These BMMϕ were then activated (with the appropriates doses of agonist) and, after the interval required in each experiment, 10^6 BMMϕ were washed twice with PBS, lysed with 1 ml Tris-HCl 25 mM, pH 7.6 containing 0.1% Triton X-100, 7 mM β-mercaptoethanol, and 9% glycerol for 20 min at room temperature and centrifuged at 90,000 x g for 30 min. The resulting supernatant was used for protein and activity measurements.

LPS from *Thiobacillus sp. IFO 14570* and from *Rhodobacter capsulatus* were purified as previously described (29,30) and LPS from *Salmonella abortus equii* was a generous gift from Dr. Galanos at the Max Planck Institut für Immunbiologie (Freiburg).

Assays of glucose-6-phosphate dehydrogenase activity were performed at room temperature by following absorbance changes at 340 nM in a Hitachi 150-20 spectrophotometer, using quartz cuvettes with 1.0 cm light path. The assay mixture contained in 1 ml: 0.1 M Tris-HCl (pH 7.6), 0.5 mM $NADP^+$ and 3 mM glucose-6-phosphate. The reaction was started by adding 100 μl supernatant. One unit (U) of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of product/min. Protein was estimated following Bradford (27), using bovine serum albumin as standard.

NO_2^- release was measured in culture supernatants using Griess's reaction (28). Briefly, an equal volume (100 μl) of Griess's reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride and 2.5% H_3PO_4) was incubated with BMMϕ supernatant (10^5 BMMϕ) for 10 min at room temperature and absorbance was measured at 550 nm in a micro ELISA reader (Merck Mod. MIOS). NO_2^- concentration (μmoles/well) was determined using $NaNO_2$ as standard.

RESULTS AND DISCUSSION

The production of nitrites by unactivated Mϕ is virtually undetectable, but increases when macrophages are activated by substances including γ-interferon or LPS (31,32). Figure 1 shows that LPS derived from *Salmonella abortus equii* induce nitrite production and glucose-6-

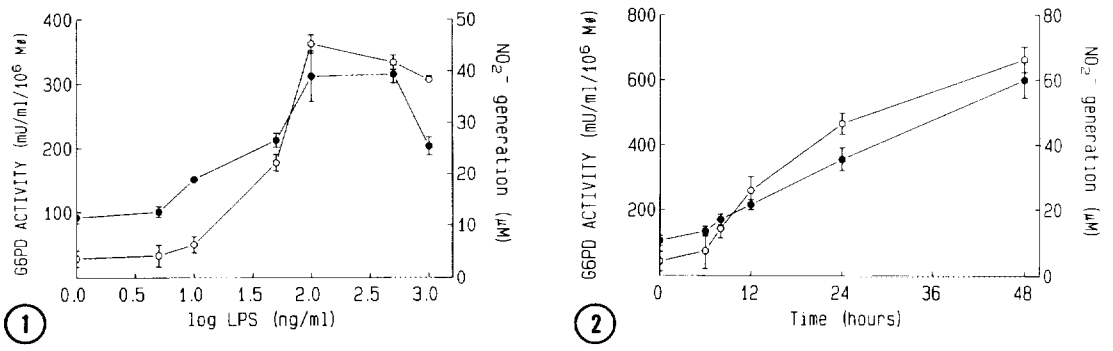

Figure 1. Dose-response curves of glucose-6-phosphate dehydrogenase activity (●) and NO_2^- generation (○) in BMMϕ activated 24 hours with LPS from *Salmonella abortus equii*.

Figure 2. Time course of glucose-6-phosphate dehydrogenase activity (●) and NO_2^- generation (○) in BMMϕ activated with LPS (0.1 μg/ml) from *Salmonella abortus equii*.

phosphate dehydrogenase synthesis to levels above baseline values in BMMϕ. At LPS concentrations greater than 0.1 μg/ml, induction ceases to be proportional, probably due to BMMϕ toxicity problems or saturation of the LPS-receptor. When measurements were made using a fixed concentration of LPS (0.1 μg/ml) but varying the incubation period (Fig. 2), an increase on glucose-6-phosphate dehydrogenase induction and NO^-_2 production was observed. The results shown in Figures 1 and 2 confirm that nitrite production is simultaneous with glucose-6-phosphate dehydrogenase induction. Given that nitric oxide synthase requires NADPH in order to produce NO· and citrulline from arginine, this result was to be expected. It may also be noticed that NADPH is used during the production of superoxide radicals; since the last takes place during the early stages of macrophage activation (33), it may conceivably lead to a depletion of this coenzyme.

Gramnegative bacterial lipopolysaccharides are known to be inducers of nitric oxide synthase (28,31). In the present study, LPS derived from the gramnegative bacteria *Salmonella abortus equii* simultaneously induced glucose-6-phosphate dehydrogenase and NO_2^-, while non toxic LPS derived from other bacteria with similar structure (29,30), such as *Thiobacillus sp. IFO 14570* or *Rhodobacter capsulatus* induced neither nitrite production nor glucose-6-phosphate dehydrogenase synthesis. Nevertheless all the employed LPS activated BMMϕ for other functions, such as respiratory burst and arginase synthesis (data not shown). Table 1 shows the results obtained in the treatment of BMMϕ with LPS from different sources. The most striking finding was that only those which induced nitrite production, recorded increased levels of glucose-6-phosphate dehydrogenase.

In order to determine whether glucose-6-phosphate dehydrogenase induction takes place at the protein synthesis level, BMMϕ were activated in the presence and in the absence of

Table 1. Glucose-6-phosphate dehydrogenase induction and NO_2^- generation in BMMϕ activated with LPS from different sources

AGONIST (μg\ml)	G6PD ACTIVITY (mU\10^6 MΦ)	NO_2^- GENERATION (μmol\10^6 MΦ)
None	89.92 ± 8.44	4.56 ± 0.67
LPS SOURCE:		
Salmonella abortus		
0.01	151.66 ± 5.68	6.19 ± 1.55
0.05	212.66 ± 11.01	22.16 ± 1.60
0.1	311.66 ± 39.32	45.24 ± 1.78
0.5	315.00 ± 13.45	41.67 ± 1.40
1.0	203.33 ± 14.22	38.31 ± 0.72
Thiobacillus sp.IFO 14570		
0.1	107.66 ± 13.71	6.22 ± 0.34
0.5	101.22 ± 7.21	5.32 ± 1.11
1.0	86.50 ± 5.83	5.43 ± 2.11
Rhodobacter capsulatum		
0.1	92.33 ± 15.30	5.12 ± 0.23
0.5	104.02 ± 7.21	5.86 ± 3.24
1.0	94.50 ± 12.50	4.89 ± 1.32

cycloheximide (table 2); no nitrite production or glucose-6-phosphate dehydrogenase induction was observed in the presence of this antibiotic. These results suggest the regulation of enzyme synthesis, rather than activity. Although glucose-6-phosphate dehydrogenase is strongly inhibited by a high NADPH/$NADP^+$ ratio (21-24), in this study $NADP^+$ substantially exceeded NADPH. Accordingly, specific activity increased from 0.37 mU/mg of protein in unactivated BMMϕ to 1.49 mU/mg of protein in BMMϕ activated for 48 hours. Eventhough nitric oxide synthase could regulate its activity by the amount of NADPH produced by glucose-6-phosphate dehydrogenase, this possibility is unliked, since nitric oxide synthase induction has been demonstrated to be at the synthesis level (see also 13,7). Nevertheless the coexistence of both types of nitric oxide synthase activity regulation of cannot be ruled out.

Mϕ malic enzyme has recently been purified (34), and the authors of that study have pointed out that this enzyme may provide nitric oxide synthase with the NADPH required for the synthesis of nitric oxide. Nevertheless it should be borne in mind that this enzyme supplies

Table 2. Inhibition by cycloheximide of glucose-6-phosphate dehydrogenase induction and NO_2^- generation in activated BMMϕ

	ACTIVATION-TIME (Hr)			
	8	12	24	48
G6PD (mU\10⁶ MΦ)				
None	109 ± 10.1	88 ± 7.44	118 ± 8.30	99 ± 12.0
LPS[a]	175 ± 7.1	215 ± 16.0	353 ± 5.13	508 ± 16.8
Cycloheximide[b]	N.D.	N.D.	107 ± 6.30	N.D.
LPS+cycloheximide[c]	96 ± 11.0	81 ± 5.57	101 ± 7.57	89 ± 11.2
NO_2^- GENERATION (μM)				
None	4.26 ± 2.82	5.2 ± 1.29	4.67 ± 2.5	5.18 ± 2.4
LPS	14.10 ± 2.86	25.9 ± 4.29	46.57 ± 3.3	66.22 ± 1.9
Cycloheximide	N.D.	N.D.	4.70 ± 1.8	N.D.
LPS+Cycloheximide	5.09 ± 0.46	4.0 ± 0.22	5.36 ± 0.4	4.43 ± 0.6

[a] LPS was added at a concentration of 0.1 μg/ml.
[b] Cycloheximide was added at a concentration of 2 μg/ml.
[c] LPS and cycloheximide were added at the same time.
N.D., Not determined.

most of the reduction equivalents required for the *de novo* synthesis of long-chain fatty acids and the authors fail to state whether or not malic enzyme is subjected to regulation at synthesis, along with nitric oxide synthase.

The results obtained in the present study suggest the possible involvement of glucose-6-phosphate dehydrogenase in the production of the NADPH required to ensure full nitric oxide synthase activity, and its possible role in the cytotoxic mechanism produced by the release of NO· from activated Mϕ. As NADPH is also required in the production of superoxided anion, inhibitors of glucose-6-phosphate dehydrogenase could also represented a step towards the desing of news substances with phamacologycal interest.

REFERENCES

1. Murphy, M.E. and Sies, H. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 10860-10864.
2. Bredt, D.S. and Snyder, S.H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 682-685.
3. Schmidt, H.H.H.W., Pollock, J.S., Nakame, M., Gorsky, L.D., Förstermann, U. and Murad, F. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 365-369.
4. Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K. and Kawai, C. (1991) J. Biol. Chem. 266, 12544-12547.
5. Hevel, J.M., White, K.A. and Marletta, M.A.1991) J. Biol. Chem. 266, 22789-22791.
6. Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.H.(1991) Nature 351, 714-718.
7. Lyons, C.R., Orloff, G.J. and Cunningham, J.M. (1992) J. Biol. Chem. 267, 6370-6374.

8. White, K.A. and Marletta, M.A. (1992) Biochemistry 61, 6627-6631.
9. Hibbs, J.B.Jr., Taintor, R.R., Vavrin, Z. and Rachlin, E.M. (1988) Biochem. Biophys. Res. Commun. 157, 87-94.
10. Moncada, S., Palmer, R.M. J. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109-142.
11. Crossin, K.L. (1991) Trends Biochem. Sci. 16, 81-82.
12. Kolb, H. and Kolb-Bachofen, V. (1992) Immunol. Today 13, 157-160.
13. Förstermann, V., Schmidt, H.H.H.W., Pollock, J.S., Sheng, H., Mitchell, J.A., Warner, T.D., Nakane, M. and Murad, F. (1991) Biochem. Pharmacol. 42, 1849-1954.
14. Mayer, B., Mathias, J., Heinzel, B., Werner, E.R., Wacher, H., Shultz, G. and Bohme, E. (1991) FEBS Lett. 288, 187-191.
15. Marletta, M.A., Yoon, P.S., Iyengar, R, Leaf, C.D and Wishnok, J.S. (1988) Biochemistry 27, 8706-8711.
16. Kather, H., Rivera, M. and Brand, K. (1972) Biochem. J. 128, 1089-1096.
17. Katz, J. and Wals, P.A. (1972) Biochem. J. 128, 879-899.
18. Gumaa, K.A., Greenbaum, A.L. and McLean, P. (1973) Eur. J. Biochem. 34, 188-198.
19. Benevenga, N.J., Stielau, W.J. and Freendland, R.A. (1964) J. Nutr. 84, 345-350.
20. Kletzien, R.F., Prostko, C.R., Stumpo, D.J., McClung, L. and Dreher, K.L. (1985) J. Biol. Chem. 260, 5621-5624.
21. Fabregat, I., Vitorica J., Satrustegui, J. and Machado, A. (1985) Arch. Biochem. Biophys. 236, 110-118.
22. Kirkman, H.N. and Gaetani, G.F. (1986) J. Biol. Chem. 261, 4033-4038.
23. Kirkman, H.N., Gaetani, G.F. and Clemons, E.H. (1986) J. Biol. Chem. 261, 4039-4045.
24. Bautista, J.M., Garrido-Pertierra, A. and Soler, G. (1988) Biochim. Biophys. Acta 967, 354-363.
25. Stuehr, D.J. and Marletta, M.A. (1987) Cancer Res. 47, 5590-5594.
26. Lucchiari, M.A., Martin, J.P., Modolell, M. and Pereira, C.A. (1991) J. Gen. Virol. 72, 1317-1322.
27. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
28. Stuehr, D.J. and Marletta, M.A. (1987) J. Immunol. 139, 518-525.
29. Mayer, H., Campos-Portuguez, S.A., Bush, M., Urbanik-Sypniewska, T. and Ramadas Bhat, U. (1990) In Cellular and molecular aspects of endotoxins reactions (A. Nowotny, J.J. Spitzer and E.J. Ziegler, eds.). pp. 111-128. Elsevier Science Publishers. Amsterdam.
30. Loppnow, H., Rietschel, E. Th., Brade, H., Schönbeck, U., Libby, P., Wang, W-F., Heine, H., Feist, W., Dürrbaum-Landmann, I., Ernst, M., Brandt, E., Grage-Griebenow, E.,Ulmer, A.J., Campos-Portuguez, S., Schade, U., Kirikae, T., Kusumoto, S., Krauss, J., Mayer, H. and Flad, H-D. (1993) In Bacterial endotoxin: Recognition and effector mechanisms (J. Levin, C.R. Alvin, R.S. Munford and P.L. Stutz, eds.). pp. 337-348. Elsevier Science Publishers. Amsterdam.
31. Stuehr, D.J. and Marletta, M.A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7738-7742.
32. Iyengar, R., Stuehr. D.J. and Marletta, M.A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 6368-6373.
33. Tsunawaki, S. and Nathan, C.F. (1984) J. Biol. Chem. 259, 4305-4312.
34. Rusche, K. M., Hevel, J.M. and Marletta, M.A. (1992) In The Biology of Nitric Oxide (S. Moncada, M.A. Marletta, J.B. Hibbs Jr. and E.A. Higgs Eds.). vol. II. pp. 45-47. Portland Press. London.