Biochemical Pharmacology, Vol. 43, No. 8, pp. 1841–1845, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

β-NAPHTHYLAMINE INDUCES ANION SUPEROXIDE PRODUCTION IN RAT PERITONEAL MACROPHAGES

M. D. CHIARA and F. SOBRINO*

Departamento de Bioquímica Médica y Biología Molecular, Facultad de Medicina, Universidad de Sevilla, Av Sanchez Pizjuan, 4, 41009-Sevilla, Spain

(*Received* 11 *May* 1991; *accepted* 16 *December* 1991)

Abstract—Rat peritoneal macrophages were incubated in the presence of β-naphthylamine (β-NA), a well known carcinogenic agent, and some parameters of respiratory burst were studied. β-NA induced a time- and dose-dependent stimulation of superoxide anion (O_2^-) production, and this enhancement was suppressed by the addition of superoxide dismutase enzyme. Also, no cooperative effect between β-NA and phorbol 12-myristate 13-acetate was observed. Other observations were as follows: (i) the simultaneous presence of polymyxin B, and staurosporine inhibitors of protein kinase C, inhibited β-NA-dependent O_2^- production; (ii) NADPH-oxidase contained in postnuclear fraction from β-NA-incubated macrophages showed a greater activity than control fractions; (iii) the stimulation of O_2^- production elicited by β-NA was several-fold enhanced in activated macrophages compared to resident cells. These data suggest that β-NA produces the activation of NADPH-oxidase through protein kinase C.

Respiratory burst is a coordinate series of metabolic events that lead to the production of oxygen-free radicals [1]. This response can be induced in phagocytic cells by exposure to appropriate stimuli which activate the O_2^- generating enzyme (NADPH-oxidase) [2]. Oxygen-free radicals may both participate in the defense mechanism against invading microbes [3] and induce damaging effects on biological molecules such as lipids [4], fibrous proteins and enzymes [5] and nucleic acids [6].

Oxygen-free radicals also have mutagenic effects. Indeed, exposure of DNA to O_2^--generating systems causes extensive strand breakage and degradation of deoxyribose [7], probably due to formation of $OH^\cdot$. Exposure of mammalian cells to activated human neutrophils produces chromosome damage and the DNA within neutrophils themselves is fragmented during phagocytosis [8]. Oxygen radicals have been suggested to be involved in the action of a number of DNA-damaging drugs. Chronic inflammation can be associated with cancer and it has been suggested that mutagenic effects of oxygen radicals produced during the respiratory burst may promote the development of cancer in chronically inflamed tissues [9].

In this paper, we have analysed whether a carcinogenic chemical such as β-naphthylamine (β-NA†) could stimulate and/or modify the oxygen-free radical production in macrophages. β-NA is an aromatic amine which causes bladder cancer [10]. Our results demonstrate that β-NA stimulated O_2^- production by peritoneal rat macrophages and suggest that this stimulation of the respiratory burst may be associated with the activation of protein kinase c.

* Corresponding author.

† Abbreviations: β-NA, β-naphthylamine; PMA, phorbol 12-myristate 13-acetate.

MATERIALS AND METHODS

Materials. Chemicals and β-NA were of analytical grade from Merck (Darmstadt, Germany). Biochemicals were from Boehringer (Mannheim, Germany) or the Sigma Chemical Co. (St Louis, MO, U.S.A.). β-NA was freshly prepared and dissolved in ethanol.

Macrophage preparation. Macrophages were obtained from peritoneal rat cells as described previously [11] based on the method of Mosier [12]. Briefly, adherent cells were collected after incubation in plastic Petri dishes at 37° in Krebs–Ringer–bicarbonate containing 10 mM glucose and 2% (w/v) bovine serum albumin under air/CO_2 (19:1).

Activated peritoneal macrophages were harvested from rats (100–150 g body weight) 7 days after the i.p. injection with 0.5 mL (5×10^8 cells) of *Escherichia coli* (K12-strain) as described in Ref. 13. The cellular viability was estimated by their ability to exclude Trypan blue and it was greater than 95% after macrophage incubation with any of the β-NA concentrations tested.

Superoxide assay. O_2^- production was estimated by measuring the superoxide dismutase inhibitable reduction of cytochrome *c* at 37° using a molar absorption coefficient of 21.1 $mM^{-1}\,cm^{-1}$ as described previously [14]. Macrophages (1×10^6 cells/mL) were suspended in Krebs–Ringer–bicarbonate, pH 7.4, containing glucose (10 mM) and cytochrome *c* (80 μM). Control cuvettes also contained superoxide dismutase (90 μg/mL). After prewarming the cells at 37°, β-NA was added and absorbance was continuously recorded at 550 nm in a Uvikon spectrophotometer equipped with an automatic cell changer.

Preparation of postnuclear supernatants from macrophages. Postnuclear supernatants were obtained from macrophages as we described previously [11]. Briefly, macrophages were incubated

with 20 mM β-NA [or 1% (v/v) ethanol in control cells] at 37° for 5 min. Reaction was stopped by placing the cells in an ice-bath. Macrophages were then collected by centrifugation, suspended in 10 mM Tris/0.35 M sucrose, pH 7.2, and homogenized with a Potter–Elvehjem homogenizer at 4°. The homogenate was centrifuged at 1000 *g* to eliminate nucleus and unbroken cells. The supernatant—termed postnuclear supernatant—was immediately used to measure NADPH-oxidase activity.

NADPH-oxidase activity. The oxidase activity was determined by measuring the superoxide dismutase inhibition of cytochrome *c* reduction at 25° as in Ref. 15. Cuvettes (1 mL) contained 10 mM phosphate buffer (pH 7.2) 100 mM NaCl, 1 mM $MgCl_2$, 80 μM cytochrome *c* and the postnuclear supernatant. The O_2^- production was started by the addition of NADPH and the absorbance change at 550 nm was followed.

Protein concentration. Protein concentration was determined by the modified Lowry method [16] with bovine serum albumin as standard.

RESULTS

Evidences of O_2^- production by rat peritoneal macrophages in the presence of β-NA

To test whether β-NA was able to stimulate the O_2^--generating enzyme, rat peritoneal macrophages were incubated in the presence of cytochrome *c* and absorbance changes were registered before and after β-NA addition as described in Materials and Methods. We observed a cytochrome *c* reduction after β-NA addition to the cells, but not after β-NA addition either in the presence of superoxide dismutase (90 μg/mL) or in the absence of cells. Redox status of the cytochrome was not modified by β-NA solvent [1% (v/v) ethanol] either. Thus, we conclude that cytochrome *c* reduction was specifically caused by its reduction with O_2^- anion, which was synthesized after the β-NA addition to the cells.

Time-course and dose-dependent O_2^- production

Figure 1 illustrates the time-dependent O_2^- production by β-NA-stimulated resident macrophages. The rate of O_2^- production was linear for 5–10 min and lasted about 30 min. A similar kinetic pattern was found when phorbol 12-myristate 13-acetate (PMA) (100 nM) was used as respiratory burst stimulus (results not shown).

The rate of O_2^- production was dependent on β-NA concentration. Figure 2 shows O_2^- production by resident and activated macrophages in response to different β-NA concentrations. Maximal stimulating β-NA concentration was 20 mM in resident macrophages and 5 mM in activated macrophages. The rate of O_2^- production in response to any β-NA concentration was about 10-fold greater in activated than in resident macrophages. It is known that macrophage differentiation to the activated state involves a 5–10-fold increase of radical oxygen production regarding resident cells [17]. Our data are in agreement with those in the literature.

NADPH-oxidase activity

NADPH-dependent O_2^- production was assayed in the postnuclear supernatant from 20 mM β-NA-stimulated resident macrophages. Control cells were incubated with the β-NA solvent [1% (v/v) ethanol]. Figure 3 shows the O_2^--forming activity as a function of NADPH concentration. A 6-fold lower NADPH-oxidase activity was found in postnuclear super-

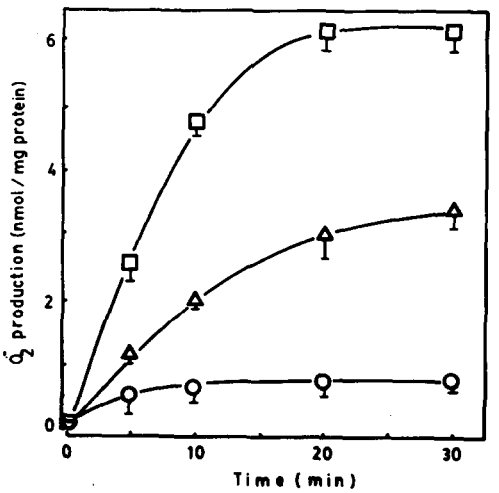

Fig. 1. Kinetics of O_2^- production by β-NA-stimulated resident macrophages. Resident peritoneal macrophages (1×10^6 cells/mL) were stimulated with 5 mM (○), 10 mM (△) and 20 mM β-NA (□). O_2^- production was measured at different times of incubation. Means ± SEM from five separate experiments performed in duplicate are presented.

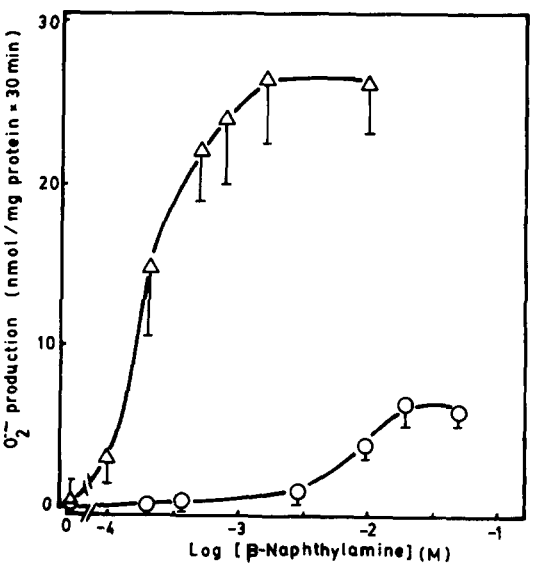

Fig. 2. Effect of β-NA concentration on O_2^- production by resident and activated macrophages. Macrophages, 1×10^6 resident (○) or activated (△), were exposed to different β-NA concentrations. O_2^- production was measured 30 min after adding β-NA. Data are means ± SEM from eight separate experiments.

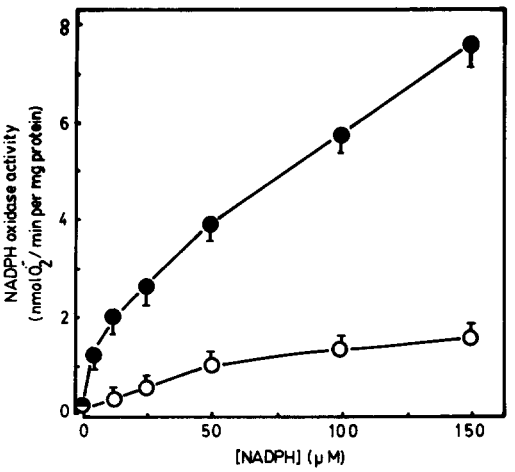

Fig. 3. NADPH oxidase activity in 20 mM β-NA-stimulated resident macrophages. Resident macrophages were incubated with 20 mM β-NA (●) or its solvent (○) for 5 min at 37°. The postnuclear supernatant was obtained from macrophages as described in Materials and Methods. Data are means ± SEM from two separate experiments performed in duplicate.

natants from 1% ethanol-treated macrophages compared with that found in cell extracts from β-NA-treated macrophages. We also found that 1%-ethanol treatment of the cells did not alter the basal oxidase activity measured in cell extracts from non-treated macrophages.

Effect of inhibitors of protein kinase C

The aim of the next experiment was to analyse the role of protein kinase C on the mechanism involved in the activation of NADPH-oxidase by β-NA. Resident macrophages were incubated with PMA and β-NA at different concentrations. Table 1 shows that maximal O_2^- production was elicited by 100 nM PMA or by 20 mM β-NA. At these concentrations, β-NA plus PMA-stimulated macrophages did not produce more O_2^- than cells stimulated independently by either of the stimuli. However, at submaximal concentration (e.g. with 50 mM PMA plus 10 mM β-NA) a cooperative effect was observed between both agents. These data could suggest that both drugs share the same mechanism of oxidase activation. To test this possibility, inhibitors of protein kinase C (polymyxin B and staurosporine) were tested simultaneously with β-NA. It is well established that protein kinase is the PMA-intracellular receptor and it seems that PMA stimulates NADPH-oxidase through the activation of protein kinase C [18]. Polymyxin B [19] and staurosporine [20] are potent inhibitors of protein kinase C and they reduce the PMA-dependent O_2^- production. Table 1 shows that 20 μg/mL polymyxin B and 10^{-8} M staurosporine reduced β-NA-dependent O_2^- production significantly. A more detailed study on the inhibitory effect of 5 μg/mL polymyxin B is illustrated in Fig. 4. The inhibition percentage remained constant (35.2%) with either of the β-NA concentrations tested, and it was greater than that observed when PMA was used as stimulus (results not shown). This rate of inhibition was increased to 50.2 and 73.4% when 10 and 25 μg/mL of polymyxin B were used, respectively. These data could indicate that the respiratory burst-stimulating effect of β-NA might be mediated by an activation of protein kinase C.

DISCUSSION

The present work provides evidence that a potent carcinogenic chemical, β-NA, is a stimulating agent of respiratory burst in macrophages. So far, the tumoral promoter, PMA, has been described as the most potent stimulating agent of respiratory burst [3, 21]. Apparently, PMA binds to and activates a phospholipid-dependent protein kinase C which is then translocated to the membrane where it activates the NADPH oxidase [22]. Features of the β-NA-stimulated respiratory burst are similar to those

Table 1. O_2^- production by resident macrophages exposed to β-NA, PMA, polymyxin B and saturosporine

Stimulant agent of respiratory burst	O_2^- production (nmol/30 min/mg protein)
PMA (20 nM)	2.60 ± 0.11
PMA (50 nM)	4.83 ± 0.12
PMA (100 nM)	7.13 ± 0.31
β-NA (5 mM)	0.34 ± 0.02
β-NA (10 mM)	3.21 ± 0.22
β-NA (20 mM)	6.42 ± 0.36
PMA (100 nM) + β-NA (10 mM)	7.25 ± 0.30
PMA (50 nM) + β-NA (10 mM)	7.39 ± 0.36
β-NA (20 mM) + polymyxin B (25 μg/mL)	1.70 ± 0.20
β-NA (20 mM) + staurosporine (10^{-8} M)	1.53 ± 0.12

Cells were incubated with PMA, β-NA, polymyxin B and staurosporine for 30 min. Data are mean ± SEM from three separate experiments.

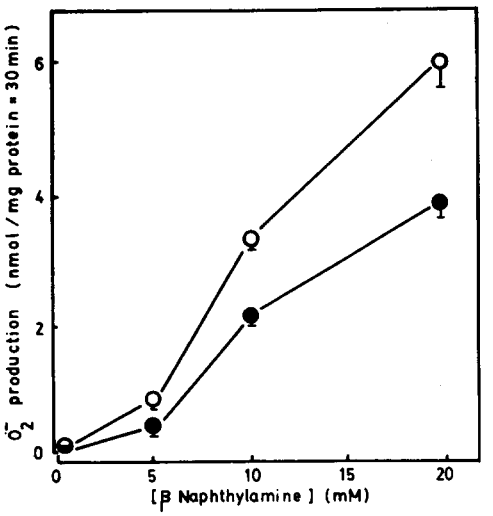

Fig. 4. Effect of polymyxin B on O_2^- production by β-NA-stimulated resident macrophages. Resident macrophages were incubated with different β-NA concentrations in the presence (●) or in the absence (○) of polymyxin B (5 μg/mL). Data are means ± SEM of three experiments performed in duplicate.

previously observed by us [11] and other authors [23, 24] when PMA was used as stimulus. These data, taken together with (i) the absence of cooperative effect between PMA and β-NA at maximal stimulating concentrations, and (ii) polymyxin B- and staurosporine-induced inhibition of β-NA-stimulated O_2^- production, strongly support that protein kinase C may be involved in the intracellular signal transduction pathway of the β-NA stimulation of O_2^- production. β-NA might directly activate protein kinase C or promote membrane phosphatidylinositol breakdown, leading to diacylglycerol production which stimulates protein kinase C.

These data demonstrate that β-NA is a respiratory burst-stimulating agent, besides being a potent carcinogenic chemical, sub-lethal damage to genetic material in inflamed foci caused by phagocyte-derived oxygen metabolites may be one mechanism by which cancer arises in the presence of inflammation. The observation that human phagocytes are important in this mutagenic process [25–27] is consistent with this idea, since there is a strong correlation between mutagenesis and carcinogenesis [28]. In addition, it has been stated that human phagocytes that are activated to produce oxygen radicals also produce cytogenic damage in cultured mammalian cells, an observation that may help to explain the relation between chronic inflammatory states and cancer *in vivo*. Xenobiotic tumour promoters can intercalate into cellular membranes and are metabolized slowly [29]. In addition, it has been suggested that phagocytic cells may play a role in PMA-induced skin tumor promotion [30]. Thus, a chronic stimulation of protein kinase C can induce a malignant transformation in the cell [9]. Although we cannot establish a direct relationship between β-NA-induced respiratory burst and carcinogenesis, our observations suggest a mechanism to interpret the DNA damage in cells chronically exposed to β-NA.

Acknowledgements—This work was supported by Consejería de Salud (Junta de Andalucía) and by the Fondo Investigaciones Sanitarias, Spain (Grant No. 812/90) and by Direccion General Investigacion Cientifica y Técnica, Spain (Grant No. PM90-154). M.D. Chiara is a recipient of predoctoral fellowships from Junta de Andalucía (Spain).

REFERENCES

1. Babior BM, Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* **298**: 659–668, 1978.
2. Pick E and Keisari Y, Superoxide anion and H_2O_2 production by chemically elicited peritoneal macrophages—induction by multiple nonphagocytic stimuli. *Cell Immunol* **59**: 301–318, 1981.
3. Badwey JA and Karnovsky ML, Active oxygen species and the functions of phagocytic leukocytes. *Annu Rev Biochem* **49**: 695–726, 1980.
4. Lynch RE and Fridovich I, Effects of superoxide on the erythrocyte membrane. *J Biol Chem* **253**: 1838–1845, 1978.
5. Berton G, Dusi S and Bellavite P, The respiratory burst of phagocytes. In: *The Respiratory Burst and its Physiological Significance* (Eds. Ibarra A J and Straus RR), pp. 33–52. Plenum Press, New York, 1988.
6. Myers LS. Free radical damage of nucleic acids and their components by ionizing radiation. *Fed Proc* **32**: 1882–1894, 1973.
7. Brawn K and Fridovich I, DNA strand scission by enzimically generated oxygen radicals. *Arch Biochem Biophys* **206**: 414–419, 1981.
8. Birnboim HC, Factors which affect DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate. *Can J Physiol Pharmacol* **60**: 1359–1366, 1982.
9. Halliwell B and Gutteridge JMC, Review article: oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219**: 1–14, 1984.
10. Boyland E, The biochemistry of aromatic hydrocarbons, amines and urethane. In: *Physico-chemical Mechanisms of Carcinogenesis* (Eds. Bergmann ED and Pullman B), pp. 25–43. The Israel Academy of Sciences and Humanities, Jerusalem, 1969.
11. Chiara MD, Bedoya F and Sobrino F, Cyclosporin A inhibits phorbol ester-induced activation of superoxide production in resident mouse peritoneal macrophages. *Biochem J* **264**: 21–26, 1989.
12. Mosier DE, Separation of macrophages on plastic and glass surfaces. *Methods Enzymol* **108**: 294–297, 1984.
13. Chiara MD and Sobrino F, Modulation of the inhibition of respiratory burst in mouse macrophages by cyclosporin A: effect of *in vivo* treatment, glucocorticoids and the state of activation of cells. *Immunology* **72**: 133–137, 1991.
14. Babior BM, Kipnes RS and Curnutte JT, Biological defense mechanisms. The production of leukocytes of superoxide. A potential bactericidal agent. *J Clin Invest* **52**: 741–744, 1973.
15. Heyneman RA and Vercauteren RE, Activation of a NADPH oxidase from horse polymorphonuclear leukocytes in a cell free system. *J Leukocyte Biol* **36**: 751–759, 1984.
16. Markwell MAK, Haas SM, Bieber LL and Tolhert NE, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**: 206–210, 1978.
17. Pabst MJ and Johnston RB, Increased production of

superoxide anion by macrophages exposed *in vitro* to muramyl dipeptide or lipopolysaccharide. *J Exp Med* **151**: 101–114, 1980.

18. Wolfson M, McPhail LC, Nasrallah VN and Snyderman R, Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: potential role in the activation of the respiratory burst enzyme. *J Immunol* **135**: 2057–2062, 1985.
19. Wise BC, Glass DB, Jen Chou C-K, Reyner RL, Katch N, Schatzman RC, Turner RS, Kibler RF and Kuo JF, Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. *J Biol Chem* **257**: 8489–8495, 1982.
20. Combadiére C, Hakim J, Giroud JP and Périanin A, Stauroporine, a protein kinase inhibitor, up-regulates the stimulation of human neutrophil respiratory burst by *N*-formyl peptides and platelet activating factor. *Biochem Biophys Res Commun* **168**: 65–70, 1990.
21. Borregaard N, The respiratory burst. An overview. In: *The Respiratory Burst and its Physiological Significance* (Eds. Ibarra AJ and Strauss RR), pp. 1–31. Plenum Press, New York, 1988.
22. Lehrer RI and Cohen L, Receptor-mediated regulation of superoxide production in human neutrophils stimulated by phorbol myristate acetate. *J Clin Invest* **68**: 1314–1320, 1981.
23. Sha'afi RI, Molski TFP, Gomez-Cambronero J and Huang CK, Dissociation of the 47-kilodalton protein phosphorylation from degranulation and superoxide production in neutrophils. *J Leukocyte Biol* **43**: 18–27, 1988.
24. Sakata A, Ida E, Tominaga M and Onoue K, Arachidonic acid acts as an intracellular activator of NADPH-oxidase in Fc receptor-mediated superoxide generation in macrophages. *J Immunol* **138**: 4353–4359, 1987.
25. Weitzman SA and Stossel TP, Mutation caused by human phagocytes. *Science* **212**: 546–547, 1981.
26. Weitzman SA and Stossel TP, Effects of oxygen radical scavengers and antioxidants on phagocyte-induced mutagenesis. *J Immunol* **128**: 2770–2772, 1982.
27. McCord JM and Fridovich I, The biology and pathology of oxygen radicals. *Ann Intern Med* **89**: 122–127, 1978.
28. McCann J and Ames BN, The Salmonella/microsome mutagenicity test: predictive value for animal carcinogenicity. In: *Origins of Human Cancer* (Eds. Hiatt HH, Watson JD and Winsten JA), pp. 1431–1450. Cold Spring Harbor Laboratory, New York, 1977.
29. Blumberg PM, Sharkey NA, Köning B, Jaken S, Leach KL and Jeng AY, In: *Cancer Cells I, The Transformed Phenotype*, pp. 245–251. Cold Spring Harbor Laboratory, New York, 1984.
30. Birnboim HC DNA strand breakage in human leukocytes exposed to a promoter, phorbol myristate acetate. *Science* **215**: 1247–1249, 1982.