Biochimica et Biophysica Acta 858 (1986) 217–220
Elsevier

BBA 70307

BBA Report

Contamination of commercial preparations of xanthine oxidase by a Ca^{2+}-dependent phospholipase A_2

Daniel A. Gamache, Lori J. Kornberg, Michael Bartolf and Richard C. Franson

Department of Biochemistry, Box 614, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298 (U.S.A.)

(Received February 24th, 1986)

Key words: Xanthine oxidase; Phospholipase A_2; Lipid peroxidation; Enzyme assay; (Contaminant)

Using [1-^{14}C]oleate-labelled autoclaved *Escherichia coli* as substrate, we demonstrate that many, but not all, commercial preparations of xanthine oxidase contain phospholipase A_2 activity as a contaminant. Phospholipase A_2 activity (64.3–545.6 nmol phospholipid hydrolyzed per min per mg protein) was optimal in the neutral to alkaline pH range, was Ca^{2+}-dependent, and was unaffected by the addition of xanthine. Phospholipase A_2 activity was totally inhibited by 1.0 mM EDTA while radical production by xanthine plus xanthine oxidase was unaffected by EDTA. Even chromatographically purified xanthine oxidase (Sigma Grade III) contained substantial phospholipase A_2 activity (64.3 nmol/min per mg). Since the preparation of xanthine oxidase employs proteolytic digestion of milk or buttermilk by pancreatin, an extract of pancreas which is an organ rich in phospholipase A_2 activity, we speculate that the contaminant phospholipase A_2 is introduced by this treatment. Because xanthine oxidase is used extensively to study free radical-induced cell injury and membrane phospholipid alterations, the presence of a potent extracellular phospholipase A_2 may have influenced previously published reports and such studies in the future should be interpreted with care.

Oxygen free radicals can oxidize and/or reduce nucleic acids, proteins, and phospholipids and, thereby, participate in the pathophysiology of cell injury [1]. One of the most widely studied biochemical consequences of oxygen free radical production is membrane phospholipid peroxidation, an autocatalytic process initiated by nucleophilic attack of fatty acid double bonds in membrane phospholipid [1,2]. Peroxidation of membrane phospholipid results in increased membrane permeability and altered enzyme function [3–5]. Since these functional changes are associated with membrane phospholipid alterations [3], activation of cellular phospholipases is thought to contribute to radical-induced membrane dysfunction [3,6]. In recent studies designed to investigate the relationship between phospholipid peroxidation and susceptibility of phospholipid to attack by cellular phospholipases, we noted significant phospholipid breakdown with some commercial preparations of xanthine oxidase. This report describes the presence of a potent Ca^{2+}-dependent phospholipase A_2 that contaminates most commercial preparations of xanthine oxidase.

Materials. Commercial xanthine oxidase preparations assayed were: Sigma Grade I (buttermilk), Sigma Grade III (buttermilk, chromatographically purified), Sigma Grade IV (milk), Calbiochem (bovine milk), and Boehringer Mannheim (cow milk). Highly purified xanthine oxidase prepared from cream by the method of Waud et al. [7] was kindly supplied by Dr. Eugene Gregory. Xanthine, cytochrome *c* (Type VI, horse heart), and superoxide dismutase (Type I, bovine

blood) were purchased from Sigma Chemical Co., St. Louis, MO.

Methods. The production of oxygen free radicals by xanthine/xanthine oxidase was determined by measuring increased absorbance of reduced cytochrome *c* at 550 nm [8]. Reduction of cytochrome *c* by xanthine/xanthine oxidase was more than 90% inhibited by the addition of 10 μg of superoxide dismutase confirming the production of superoxide. Each preparation of xanthine oxidase was diluted 1 : 9 (v/v), and 20 μl was then added to the standard reaction mixture. Activity is expressed as ΔA at 550 nm per min per mg protein. Protein was determined by the method of Bradford [9] using ovalbumin as a standard.

Phospholipase A_2 activity was routinely assayed using autoclaved Escherichia coli whose phospholipids were labelled almost exclusively in the 2-acyl position during growth with [1-^{14}C] oleate [10]. To confirm positional specificity of the phospholipase A, 1-[1-^{14}C]stearoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine was used as substrate [10]. Reaction mixtures contained 1.0 mM $CaCl_2$ (unless indicated otherwise), 25 mM buffer (acetate, pH 4.0–5.5; Bis Tris, pH 5.5–7.5; Tris-HCl, pH 7.5–9.0), autoclaved *E. coli* (8000 cpm and 14.3 nmol phospholipid), and 15 μl of a 1000-fold dilution of xanthine oxidase in a total volume of 0.5 ml. Xanthine oxidase preparations lacking activity were assayed without dilution. Reaction mixtures were incubated at 37°C for 10 min and the reactions were stopped by adding 3.0 ml of chloroform/methanol (1 : 2, v/v). The lipids were extracted and separated as previously described [10]. Phospholipase A_2 activity is expressed as nmols phospholipid hydrolyzed per min per mg protein or as percent of maximal activity. All data are the average of duplicate or triplicate determinations using concentrations of xanthine oxidase with activity in the linear range of the assay, and are corrected for non-enzymatic hydrolysis ($\leq$ 1.4% in all experiments).

Results. When commercial preparations of xanthine oxidase (Sigma Grades I and III) were incubated with radiolabelled *E. coli* to peroxidize phospholipid, [^{14}C]oleate was released from microbial phospholipids in a time- and protein-dependent manner with or without added xanthine (not shown). This suggested the presence of phospholipid-splitting activity in commercial preparations of xanthine oxidase. Table I and Fig. 1 illustrate the conditions for optimal phospholipase A_2 activity in Sigma Grade III, chromatographically purified xanthine oxidase. Phospholipase A_2 activity was optimal in the neutral-to-alkaline pH range (Fig. 1), was Ca^{2+}-dependent and totally inhibited by 1.0 mM EDTA, and was not influenced by the presence of xanthine (Table I). Since incubation of xanthine oxidase with 1-[1-^{14}C]stearoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine released only [^{14}C]lysophosphatidylethanolamine, the contaminant phospholipase A is specific for the 2-position.

TABLE I

CALCIUM DEPENDENCE OF XANTHINE OXIDASE-ASSOCIATED PHOSPHOLIPASE A_2

Phospholipase A_2 activity was measured with Sigma Grade III xanthine oxidase as described in Methods. Data are the mean of duplicate determinations ± S.E., $n \geq 3$, n.d. = not detectable, 100% (10 mM $CaCl_2$) activity = 64.3 ± 9.1 nmol/min per mg.

Addition	Phospholipase A_2 (% of maximal)
None	22.7 ± 1.0
EDTA (1.0 mM)	n.d.
$CaCl_2$ (25 μM)	59.1 ± 8.5
$CaCl_2$ (100 μM)	68.2 ± 7.3
$CaCl_2$ (1.0 mM)	76.1 ± 1.1
$CaCl_2$ (10.0 mM)	100.0
Xanthine (50 μM)	22.7 ± 1.3

Table II compares the Ca^{2+}-dependent phospholipase A_2 activity of various preparations of

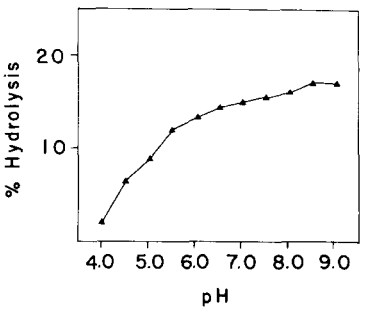

Fig. 1. Phospholipase A_2 activity of xanthine oxidase (Sigma Grade III) as a function of pH. Values represent the mean of duplicate determinations.

TABLE II

RADICAL PRODUCTION AND PHOSPHOLIPASE A_2 ACTIVITY OF XANTHINE OXIDASE PREPARATIONS

Values represent the mean ± S.E. of triplicate determinations at pH 7.0, $n \geq 3$, n.d. = not detectable.

Source	Cytochrome *c* reduction (ΔA_{550}/min per mg)	Phospholipase A_2 activity (nmol/min per mg)
Sigma Grade I	1.21 ± 0.03	545.6 ± 38.3
Sigma Grade III	0.36 ± 0.05	64.3 ± 9.1
Sigma Grade IV	0.18 ± 0.01	149.8 ± 15.1
Calbiochem	0.71 ± 0.02	251.0 ± 35.6
Boehringer Mannheim	0.60 ± 0.01	n.d.
Highly purified [7]	0.13 ± 0.02	n.d.

xanthine oxidase with that of oxygen free radical production. All preparations reduced cytochrome *c* in the presence of xanthine (0.13–1.21 ΔA_{550}/min per mg). This activity was 90% inhibited by the addition of 10 μg of superoxide dismutase and was unaffected by a concentration of EDTA (1.0 mM) that abolished phospholipase A_2 activity (Table I). All commercial preparations of xanthine oxidase, except that from Boehringer Mannheim, contained substantial phospholipase A_2 activity (64.3–545.6 nmol/min per mg). In crude preparations of xanthine oxidase the ratio of phospholipase A_2 activity to radical production ranged from 353 to 832 and was only slightly reduced (179) in chromatographically purified xanthine oxidase (Sigma Grade III). Xanthine oxidase purified from cream by the method of Waud et al. [7] was devoid of phospholipase A_2 activity.

Discussion. These studies demonstrate that most commercial preparations of xanthine oxidase contain substantial Ca^{2+}-dependent, neutral-to-alkaline-active phospholipase A_2 activity (64.3–545.6 nmol phospholipid hydrolyzed per min per mg protein). Even chromatographically purified xanthine oxidase (Sigma Grade III) had phospholipase A_2 activity (64.3 nmol/min per mg) that was readily detectable well below concentrations of xanthine oxidase ($1.4 \cdot 10^{-3}$ units/ml) used to peroxidize membrane phospholipid [11,12]. Xanthine oxidase from Boehringer Mannheim prepared by tryptic digestion of cow milk was devoid of phospholipase A_2 activity as was a highly purified xanthine oxidase prepared without proteolytic digestion [7]. Commercial preparation of xanthine oxidase commonly employs proteolytic digestion of milk or buttermilk with pancreatin [13,14]. Pancreatin is a crude, organic solvent extract of pancreas known to be rich in trypsin, chymotrypsin, and carboxypeptidase. These proteolytic enzymes, which digest milk protein in the preparation of xanthine oxidase, are contaminants of commercial xanthine oxidase preparations [14,15]. Pancreatin is also the likely source of xanthine oxidase-associated phospholipase A_2 since the pancreas is enriched in this enzyme. The properties of purified pancreatic phospholipase A_2 resemble those of xanthine oxidase-associated phospholipase A_2 in terms of positional specificity, Ca^{2+}-dependency, and pH optimum [16]. Highly purified pancreatic phospholipase A_2 hydrolyzes exogenous phosphatidylcholine [15] and autoclaved *E. coli* (unpublished observation) at approximately 450–600 μmol/min per mg. While phospholipase A_2 activity associated with xanthine oxidase preparations is considerably lower (0.06–0.5 μmol/min per mg) than the purified pancreatic enzyme, this activity is 100–1000-fold greater than measurable phospholipase A_2 activity in tissue and cell homogenates from various mammalian sources [10,17,18].

Exogenously added phospholipase A_2 produces changes in the phospholipid content of myocardial membranes [19] and alterations in the function of biological membranes [20]. Thus, Chan et al. [3,21] reported that xanthine (hypoxanthine)/xanthine oxidase peroxided lipids and increased the permeability of membranes in rat brain cortical slices. Associated with these perturbations was the release of unsaturated fatty acids. This increased lipase activity was ascribed to activation of endogenous phospholipase A_2 by oxygen free radicals but could be due to exogenous phospholipase A_2 contaminating the xanthine oxidase preparation. In their study, it is not possible to assess the contribution of exogenous phospholipase A_2 since a xanthine oxidase control was not reported [3]. However, effects of xanthine oxidase on membrane function that are xanthine-independent have been reported. Xanthine oxidase alone perturbed

the $(Na^+ + K^+)$-ATPase of cultured endothelial cells [22] and induced the aggregation and release reaction of human platelets [23]. Thus, in addition to the well known contribution of proteolytic contaminants, phospholipase A_2 may contribute to substrate-independent effects of commercial xanthine oxidase. Future studies of oxygen free radical-induced cell membrane dysfunction using xanthine oxidase should be interpreted with caution and preferably done with phospholipase-free preparations of xanthine oxidase.

The authors wish to thank Dr. Eugene Gregory of the Department of Biochemistry and Nutrition at Virginia Polytechnical Institute and State University, Blacksburg, VA, for kindly providing the highly purified xanthine oxidase preparation used in these studies. Supported by a grant from NIH AM34558 and a fellowship from the American Heart Association/Virginia Affiliate.

References

1 Halliwell, B. and Gutteridge, J.M.C. (1982) in Advances in Studies on Heart Metabolism (Caldavera, C.M. and Harris, P., eds.), pp. 403–411, CLUEB, Bologna
2 Plaa, G.L. and Witschi, H. (1976) Annu. Rev. Pharmacol. 16, 125–141
3 Chan, P.H., Yurko, M. and Fishman, R.A. (1982) J. Neurochem. 38, 525–531
4 Hess, M.l., Okabe, E. and Kontos, H.A. (1981) J. Mol. Cell. Cardiol. 13, 767–772
5 Baba, A., Lee, E., Ohta, A., Tatsuno, T. and Iwata, H. (1981) J. Biol. Chem. 256, 3679–3684
6 Weglicki, W.B., Dickens, B.F. and Mak, I.T. (1984) Biochem. Biophys. Res. Commun. 124, 229–235
7 Waud, W.R., Brady, F.O., Wiley, R.D. and Rajagopalan, K.V. (1975) Arch. Biochem. Biophys. 169, 695–701
8 McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055
9 Bradford, M. (1976) Anal. Biochem. 72, 248–254
10 Franson, R.C. and Weir, D.L. (1982) Lung 160, 275–284
11 Tein, M., Svingen, B.A. and Aust, S.D. (1982) Arch. Biochem. Biophys. 216, 142–151
12 Miura, T., Sakurai, K. and Ogiso, T. (1984) Chem. Pharm. Bull. 32, 3227–3234
13 Gilbert, D.A. and Bergel, F. (1964) Biochem. J. 90, 350–353
14 Ager, A. and Gordon, J.L. (1984) Biochem. J. 222, 839
15 Sigma Chemical Company, St. Louis, MO (1985) Product Catalog
16 DeHaas, G.H., Postema, N.M., Nieuwenhuizen, W. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 159, 103–117
17 Franson, R.C., Weiss, J., Martin, L., Spitznagel, J.K. and Elsbach, P. (1977) Biochem. J. 167, 839–841
18 Jesse, R.L. and Franson, R.C. (1979) Biochim. Biophys. Acta 575, 467–470
19 Owens, K., Pang, D.C., Franson, R.C. and Weglicki, W.B. (1980) Lipids 15, 534–538
20 Lefkowitz, R.J. (1975) J. Mol. Cell. Cardiol. 7, 27–37
21 Chan, P.H., Fishman, R.A., Schmidley, J.W. and Chen, S.F. (1984) J. Neurosci. Res. 12, 595–605
22 Ager, A., Wenham, D.J. and Gordon, J.L. (1984) Thromb. Res. 35, 43–52
23 Ishii, H., Hiraishi, S. and Kazama, M. (1982) Thromb. Res. 28, 85–92