FEBS Letters 348 (1994) 65–69

FEBS 14163

Free radical activation of acetaldehyde and its role in protein alkylation

Emanuele Albano[a,*], Paolo Clot[b], Adriana Comoglio[b], Mario U. Dianzani[b], Aldo Tomasi[c]

Departments of [a]Medical Sciences and [b]Experimental Medicine and Oncology, University of Turin, Novara, Italy
[c]Institute of General Pathology, University of Modena, Modena, Italy

Received 9 May 1994

Abstract

The formation of carbon centered free radicals, identified as methylcarbonyl species, was observed using ESR spectroscopy and the spin trapping agent 4-pyridyl-1-oxide-*N*-*t*-butyl nitrone (4-POBN) during the oxidation of acetaldehyde by xanthine oxidase. The reaction was dependent upon the presence of OH• radicals and was inhibited by the addition of superoxide dismutase, catalase or OH• radical scavengers. The generation of methylcarbonyl radicals was associated with a doubling of stable acetaldehyde adducts with serum albumin, and 4-POBN or superoxide dismutase and catalase, completely blocked this effect. Thus, methylcarbonyl radicals contributed to acetaldehyde-mediated protein alkylation which is involved in causing toxic as well as immunological reactions ascribed to acetaldehyde.

Key words: Acetaldehyde; Free radical; Spin trapping; Xanthine oxidase

1. Introduction

Acetaldehyde, the main product of ethanol oxidation, is believed to play an important role in the adverse effects of ethanol [1]. Acetaldehyde has been demonstrated to link with several proteins [2], and to interfere with enzymatic and cellular functions [3,4]. Moreover, acetaldehyde-bound proteins can act as neoantigens, promoting immunological responses in experimental animals and in patients with alcoholic liver disease [5–7].

The possible involvement of free radical species in mediating some of the damaging effects of alcohol abuse is now receiving increasing attention in relation to the observation that, in experimental animals and in humans, chronic ethanol exposure is associated with the formation of free radical intermediates and with the occurrence of oxidative stress [8–10].

Recent studies have shown that the oxidation of acetaldehyde by xanthine oxidase (EC 1.2.3.2) or aldehyde oxidase (EC 1.2.3.1) results in the stimulation of lipid peroxidation in both membrane systems [11] and isolated hepatocytes [12]. Reactive oxygen species such as superoxide anion, hydroxyl radical (OH•) and hydrogen peroxide are produced during the oxidation of acetaldehyde by xanthine oxidase [11,13]. Furthermore, Puntarulo and Cederbaum have reported that the interaction of OH• with acetaldehyde is responsible for the development of chemiluminescence during acetaldehyde oxidation by xanthine oxidase [14].

We have previously observed that alcohol-derived radicals originate from interactions with OH• [8] and that hydroxyethyl radicals are able to covalently bind to proteins [15]. These findings have prompted us to investigate whether carbon-centered free radicals might be produced during xanthine oxidase-mediated oxidation to acetaldehyde and contribute to the alkylation of proteins caused by the aldehyde [2].

2. Materials and methods

NAD^+, bakers yeast alcohol dehydrogenase, fatty acid-free bovine serum albumin, cow milk xanthine oxidase, allopurinol, catalase, superoxide dismutase and ferritin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The spin trap 4-pyridyl-1-oxide-*N*-*t*-butyl nitrone (4-POBN) was purchased from Aldrich Europe (Bersee, Belgium). [^{14}C]Ethanol was supplied by Amersham International plc (Amersham, UK). All other chemicals were of analytical grade and were purchased from Merch (Darmstadt, Germany).

The formation of acetaldehyde-derived radicals was studied using an incubation mixture containing 50 mM Na-phosphate buffer, pH 7.4, 50 μM $FeCl_3$, 0.1 mM EDTA, 0.2 U/ml xanthine oxidase, various concentrations of acetaldehyde and 20 mM 4-POBN as a spin trap. Alternatively, acetaldehyde was produced in the same incubation mixture from 1 mM ethanol by the action of 30 IU/ml alcohol dehydrogenase, 5 mM NAD^+. The incubation was terminated by transferring the samples to 1.5 ml Eppendorf tubes containing 0.5 ml of a chloroform/methanol (2:1 v/v) mixture as previously reported [16]. The chloroform phase was separated by centrifugation and used for ESR analysis. The ESR spectra were recorded at room temperature using a Brucker D 200SRC spectrometer with the following instrument setting: microwave power 10 mW; modulation frequency 100 kHz; modulation amplitude 1 G; field scan 100 G.

The covalent binding of acetaldehyde-derived radicals to proteins was studied by incubating bovine serum albumin (BSA) (3 mg/ml) for 30 min at 25°C in an incubation mixture (1 ml final volume) containing 50 mM Na-phosphate buffer, pH 7.4, 30 IU/ml alcohol dehydrogenase (ADH), 5 mM NAD^+, 0.2 U/ml xanthine oxidase, 50 μM $FeCl_3$, 0.1 mM EDTA and 1 mM [^{14}C]ethanol (specific activity 250 μCi/mmol). Tubes without alcohol dehydrogenase were incubated as blanks and negligible radioactivity binding was recovered under these conditions. In some experiments 20 mM 4-POBN or 500 U/ml superoxide dismutase and 300 U/ml catalase were added to the incubation mixture. At the end of the incubation the unbound acetaldehyde was removed as described by Donohue et al. [17]. Briefly, the incubation mixture was

*Corresponding author. *Address:* Dipartimento di Scienze Mediche, Università di Torino, Via Solaroli 17, 28100 Novara, Italy.
Fax: (39) (321) 620 421.

SSDI 0014-5793(94)00549-B

added to 2 vols. of ice-cold 15% trichloroacetic acid (TCA) solution containing 1.5% phosphotunstic acid (PTA). The albumin was separated by centrifugation and washed, with intervening centrifugations, once with 5 ml 10% TCA, twice with 5 ml 0.25% HCl dissolved in acetone, and then redissolved in distilled H_2O. The BSA solution was reprecipitated by the addition of a mixture of 15% TCA and 1.5% PTA and the pellet washed once with 10% TCA and twice with 5 ml of a mixture containing equal volumes of methanol-ethyl ether and chloroform. The remaining protein pellet was dried in air and subsequently solubilized in 0.25 ml 1 N NaOH and aliquots of 10 μl used for protein determination according to Peterson [18]. The protein solution was finally added to 5 ml Aquasol scintillation fluid (New England Nuclear Co., Boston, MA) and radioactivity counted with a 1217 Rackbeta scintillation counter (LKB Wallak, Uppsala, Sweden).

3. Results

The incubation of a reaction system consisting of 0.2 U/ml xanthine oxidase, 1 mM acetaldehyde, 0.1 mM EDTA and 50 μM $FeCl_3$ dissolved in 50 mM phosphate buffer, pH 7.4, with 20 mM of the spin trapping agent 4-POBN resulted in the formation of a well-defined ESR spectrum (Fig. 1; trace a). The hyperfine splitting constants of the nitroxide adducts were aN = 15.05 G, aH = 3.90 G. No ESR signal was detectable after omitting acetaldehyde from the incubation mixture (Fig. 1; trace b) or when 1 mM allopurinol, an inhibitor of xanthine oxidase, was added (Fig. 1; trace c). Conversely, the intensity of the ESR signals increased linearly with the amount of xanthine oxidase present in the system, indicating that the free radical formation was strictly dependent upon the enzyme activity (Fig. 2a). The trapping of free radicals also increased linearly with the concentration of acetaldehyde present in the reaction mixture up to 1 mM acetaldehyde (Fig. 2b). Interestingly, detectable ESR signals were evident with concentrations of acetaldehyde as low as 0.1 and 0.25 mM (Fig. 2b). These concentrations are close to the estimated levels of acetaldehyde present in the liver during ethanol metabolism [19]. We have also observed that the acetaldehyde formed during the oxidation of 1 mM ethanol by alcohol dehydrogenase (ADH) (1 μg/ml) in the presence of 5 mM NAD^+ is a substrate for xanthine oxidase (Fig. 1; trace d) and produced ESR spectra identical to those observed when the preformed aldehyde was used: no ESR signal was detectable after omitting ADH from the reaction mixture (Fig. 1; trace e).

In order to obtain further information about the nature and origin of the radical species observed, further experiments were performed using 90% ^{13}C-enriched acetaldehyde, both carbons being labelled. The presence of the paramagnetic ^{13}C nucleus in the spin adduct would result in an 18 line spectrum, which would allow the unambiguous assignment of the adduct. However, upon incubation of 1 mM [^{13}C]acetaldehyde with xanthine oxidase and 4-POBN the resulting ESR spectrum still consisted of a triplet of doublets, although the spectral lines were slightly broader (apparent aH = 4.2 G) than those obtained using [^{12}C]acetaldehyde (Fig. 3; trace a). By subtracting the spectra obtained with either [^{12}C]acetaldehyde and [^{13}C]acetaldehyde a 12 line spectrum was obtained (Fig. 3; trace b). Although the spectrum was poorly resolved, it demonstrated that ^{13}C-labelled free radicals were trapped and allowed an estimation of ^{13}C hyperfine constants ($a^{13}C$ = 4.2). Computer simulation using a mixture of ^{12}C and ^{13}C with a relative area of 15% and 85%, respectively, and hyperfine splitting constants for the [^{12}C]adduct aN = 14.98 G and aH = 3.82 G and for the [^{13}C]adduct aN = 14.98 G, aH = 3.82 G and $a^{13}C$ = 4.2 G, gave the spectrum shown in Fig. 3; trace c. For comparison, trace d of the same figure shows the simulation spectrum obtained using exclusively the ^{13}C-labelled parameters. The simulation results confirmed that under our conditions a ^{13}C-acetaldehyde adduct with 4-POBN was indeed formed and that the poorly resolved ^{13}C hyperfine splitting constants were due to the partial overlapping of the hydrogen and ^{13}C hyperfine features.

It is known that the presence of iron contributes to the generation of hydroxyl radicals ($OH^\bullet$) from superoxide anions released by xanthine oxidase [20,21]. We have observed that the omission of Fe^{3+}-EDTA decreases the formation of acetaldehyde-derived free radicals by about 70% (Table 1). No significant variation in the intensity

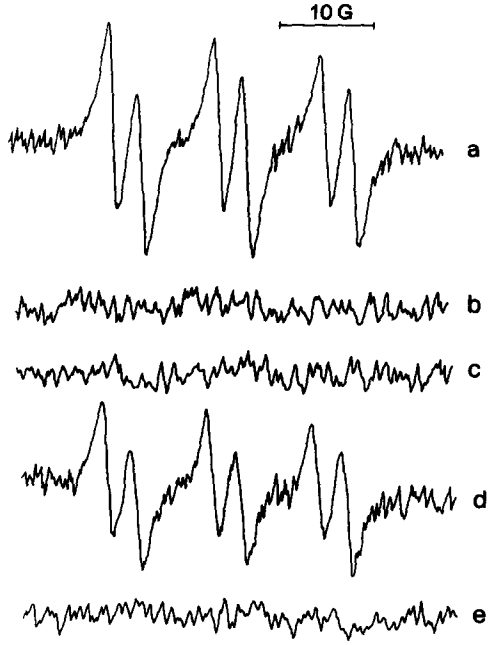

Fig. 1. ESR spectra of the 4-POBN adducts produced during the oxidation of acetaldehyde by xanthine oxidase. (Trace a) Complete reaction mixture consisting of 50 mM Na-phosphate buffer, pH 7.4, 50 μM $FeCl_3$, 0.1 mM EDTA, 0.2 U/ml xanthine oxidase, 1 mM acetaldehyde and 20 mM 4-POBN as a spin trap. (Trace b) As in (a), but without acetaldehyde. (Trace c) As in (a), plus 1 mM allopurinol. (Trace d) Reaction mixture as in (a), where acetaldehyde was produced from 1 mM ethanol by the action of 0.4 IU/ml alcohol dehydrogenase and 5 mM NAD^+. (Trace e) As in (d), but without alcohol dehydrogenase. The recorder gain was 10^6 for all the traces.

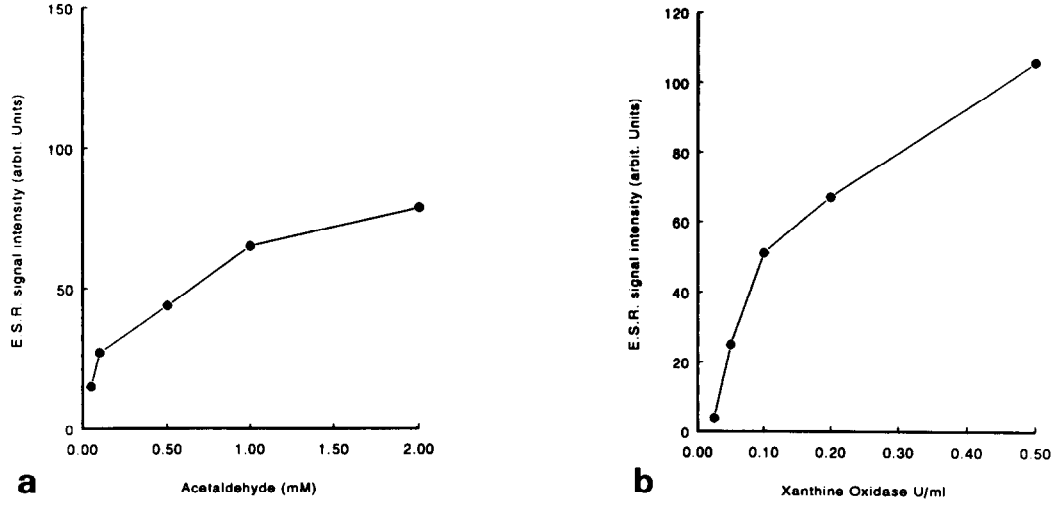

Fig. 2. Change in the intensity of the ESR signals due to the trapping of the acetaldehyde-derived free radical by 4-POBN in relation to the concentrations of, respectively, acetaldehyde (right panel) and xanthine oxidase (left panel) added to the reaction system.

of the ESR signals was appreciable, however, when $FeCl_3$ was replaced with 1 mg/ml ferritin (Table 1), probably due to the ability of superoxide anion to release iron atoms from ferritin itself [11]. Consistently, the addition of 0.1 mM desferrioxamine lowered the free radical trapping in the presence of ferritin by 50% (Table 1). The possible involvement of $OH^{\bullet}$ in the formation of acetaldehyde-derived radicals was suggested by the effect exerted by the $OH^{\bullet}$ scavengers benzoate and mannitol and by the action of superoxide dismutase and catalase that decreased the radical trapping by, respectively, 80% and 67% (Table 1). No ESR signal was detectable when superoxide dismutase and catalase were used in combination (Table 1).

The possibility that free radical intermediates might contribute to the covalent binding of acetaldehyde residues to proteins was also investigated. The generation of acetaldehyde from ^{14}C-labelled ethanol in the presence of ADH and NAD^+, resulted in the formation of stable adducts with BSA (Fig. 4). Upon incorporation of 0.2 U/ml xanthine oxidase into the reaction mixture (in order to generate acetaldehyde-derived radicals) the recovery of radioactivity covalently bound to BSA was almost doubled (Fig. 4). Such a stimulation was abolished by the addition of superoxide dismutase and catalase (Fig. 4). Furthermore, the scavenging of acetaldehyde-derived radicals by the addition of 20 mM 4-POBN completely prevented the increase in the radioactive binding caused by xanthine oxidase without affecting that occuring in the absence of the enzyme (Fig. 4). Taken together these results indicated that free radical intermediates originating from acetaldehyde were capable of alkylating proteins.

Table 1
Effect of various agents that interfere with hydroxyl radical ($OH^{\bullet}$) formation on the generation of acetaldehyde-derived free radical by xanthine oxidase

Additions	ESR signal intensity (arbitrary units)	Change (%)
None	63 ± 3	0
− Fe^{3+}-EDTA	22 ± 5	− 73
+ Superoxide dismutase 500 U/ml	15 ± 7	− 76
+ Catalase 300 U/ml	25 ± 4	− 60
+ Superoxide dismutase + catalase	3 ± 2	− 95
+ Mannitol 2.5 mM	34 ± 11	− 46
+ Na-benzoate 2.5 mM	36 ± 9	− 43
+ Ferritin 1 mg/ml	62 ± 6	− 2
+ Ferritin + desferrioxamine 0.1 mM	31 ± 2	− 50

The various agents were added to a reaction mixture containing 50 mM Na-phosphate buffer, pH 7.4, 50 μM $FeCl_3$, 0.1 mM EDTA, 0.2 U/ml xanthine oxidase, various concentrations of acetaldehyde and 20 mM 4-POBN. In some experiments ferritin (1 mg/ml) replaced $FeCl_3$ as the source of iron. The results are means of three different experiments ± S.D.

4. Discussion

It is well known that a number of aldehydes, including acetaldehyde, can be oxidized by xanthine oxidase concomitantly with the reduction of oxygen to superoxide anion and hydrogen peroxide [11–13,22]. Furthermore, highly reactive hydroxyl radicals can originate as a result of the enzyme activity [20,21].

By using ESR spectroscopy coupled with the spin trapping technique we have observed that carbon-centered radicals are also produced during the oxidation of acetaldehyde by xanthine oxidase. The origin of this radical from acetaldehyde is suggested by a dose-dependent increase in the intensity of the ESR signals with the concentration of acetaldehyde added to the system. Moreover, identical ESR spectra are detectable when xanthine oxidase acts on acetaldehyde directly generated from ethanol by alcohol dehydrogenase.

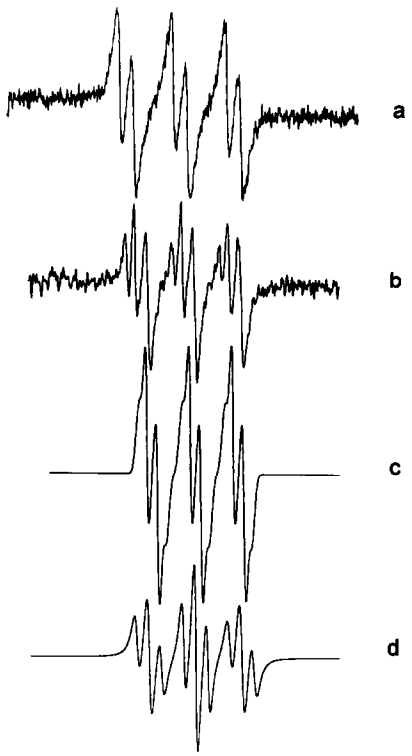

Fig. 3. ESR spectra of the 4-POBN adducts produced during the oxidation of ^{13}C-labelled acetaldehyde by xanthine oxidase. (Trace a) Complete reaction mixture consisting of 50 mM Na-phosphate buffer, pH 7.4, 50 μM $FeCl_3$, 0.1 mM EDTA, 0.2 U/ml xanthine oxidase, 1 mM [^{13}C]acetaldehyde and 20 mM 4-POBN. (Trace b) Spectrum obtained by electronic subtraction of an ESR spectrum produced during the oxidation of, respectively, 1 mM [^{13}C]acetaldehyde and [^{12}C]acetaldehyde. (Trace c) Computer simulation of an ESR spectrum obtained using a mixture of ^{12}C and ^{13}C with a relative area of 15% and 85%, respectively, and hyperfine splitting constants for the ^{12}C-adduct of aN = 14.98 G and aH = 3.82 G and for the ^{13}C-adduct of aN = 1498 G, aH = 3.82 G and $a^{13}C$ = 4.2 G. (Trace d) Computer simulation of an ESR spectrum obtained using the ^{13}C parameters only.

The formation of acetaldehyde-derived radicals appears to depend upon the presence of trace iron and is greatly decreased in the absence of Fe^{3+}-EDTA. On the other hand, ferritin can act as an efficient source of iron in agreement with previous observations showing that catalytically active iron ions are released from ferritin by the action of reactive oxygen species formed during the oxidation of acetaldehyde by xanthine oxidase [11]. The formation of acetaldehyde-derived radicals is also inhibited by the addition of superoxide dismutase and catalase or in the presence of the OH• radical scavengers mannitol and benzoate, indicating the involvement of OH• generated from superoxide by an iron-catalyzed Haber–Weiss reaction. Thus we suggest the possibility that acetaldehyde might act at the same time as the source of reactive oxygen species, being a substrate for xanthine oxidase, as well as a target for OH• radicals.

It is known that the reaction of aldehydes with OH• results primarily in α-hydrogen abstraction [23]; thus the interaction of OH• with acetaldehyde would yield the methylcarbonyl radical. It is possible, however, that this radical might be further degraded by decarboxylation before reacting with the 4-POBN, thus leading to the trapping of the methyl radical. The comparison of hyperfine splitting constants of our spectra with those of methyl-4-POBN adducts analyzed under similar conditions (aN = 14.98 G; aH = 2.46 G) [24] excludes this possibility. Such an interpretation is supported by the recovery of radioactive carbonyl atoms bound to albumin when acetaldehyde labelled by ^{14}C on the carbonyl atom was generated by oxidation of [1-^{14}C]ethanol and further metabolized by xanthine oxidase. On this basis we ascribe the radical species detected during the oxidation of acetaldehyde by xanthine oxidase to the trapping of the methylcarbonyl radical. Consistently, Puntarulo and Cederbaum have reported that the chemiluminescence produced by acetaldehyde in a reaction system containing xanthine oxidase and Fe^{3+}-EDTA does not involve the presence of products derived from methyl radicals [14].

Acetaldehyde is known to react with a variety of proteins to produce both stable and unstable adducts [2]. Unstable adducts are due to the formation of a Schiff-base with amino groups, while the structure and the reactions leading to stable acetaldehyde protein adducts have not yet been elucidated [2]. We have observed that, when acetaldehyde labelled with ^{14}C on the carbonyl atom is generated enzymatically from radioactive ethanol, the presence of xanthine oxidase increases by two-fold the formation of stable acetaldehyde adducts with BSA. The treatments that suppress the formation of

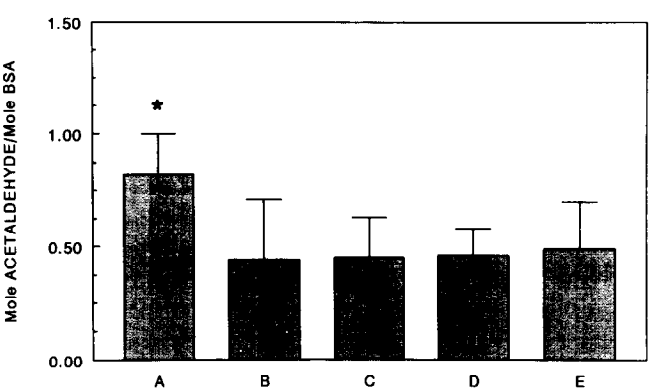

Fig. 4. Effect of xanthine oxidase on the covalent binding of radioactive acetaldehyde residues to BSA when acetaldehyde was formed from [^{14}C]ethanol by alcohol dehydrogenase in the presence of NAD^+. BSA (3 mg/ml) was incubated for 30 min at 25°C in 50 mM Na-phosphate buffer, pH 7.4, containing 30 IU/ml alcohol dehydrogenase, 5 mM NAD^+, 50 μM $FeCl_3$, 0.1 mM EDTA and 1 mM [^{14}C]ethanol (specific activity 250 μCi/mmol) in the presence (A) or in the absence (B) of 0.2 U/ml xanthine oxidase, and with xanthine oxidase plus 20 mM 4-POBN (C) or 500 U/ml superoxide dismutase and 300 U/ml catalase (D). The effect of 4-POBN on the radioactive binding in the absence of xanthine oxidase is shown in E. The values are means of three different experiments in duplicate. *Significantly different from B ($P < 0.005$) by Student's t-test.

methylcarbonyl free radicals, like the addition of 4-POBN which scavenges these radicals, completely prevent the increased binding of acetaldehyde. We have recently reported that 4-POBN also prevents the binding of hydroxyethyl radicals to microsomal proteins [15]. Therefore, we suggest that methylcarbonyl free radicals might be implicated in the alkylation of proteins.

Concerning the biological relevance of xanthine oxidase-mediated free radical activation of acetaldehyde, it has been reported that ethanol intoxication increases xanthine oxidase activity in the liver by favouring the conversion of the enzyme from the dehydrogenase to the oxidase form [25]. Furthermore, Shaw and Jayatilleke have suggested that xanthine oxidase can contribute to oxidative damage caused during ethanol intoxication [26]. Although it can be argued that the K_m of xanthine oxidase for acetaldehyde is over 30 mM [27], we have observed detectable free radical formation with amounts of acetaldehyde as low as 0.1–0.25 mM, which are in the range of the concentrations achieved in the liver following ethanol intoxication [19]. Similar findings have also been reported by Puntarulo and Cederbaum concerning the development of chemiluminescence [14]. It should be pointed out that, besides xanthine oxidase, molybdenum-containing aldehyde oxidase, which has a much lower K_m for acetaldehyde (1 mM), has also been shown to produced superoxide anion [28,29], as well as chemiluminescence, when oxidizing acetaldehyde [30]. Aldehyde oxidase has also been shown to play an important role in stimulating lipid peroxidation in isolated hepatocytes exposed to low concentration of acetaldehyde [12]. Thus, this latter enzyme could also play a role in the formation of methylcarbonyl radicals.

In conclusion, the results obtained demonstrated that acetaldehyde can be activated to a free radical intermediate when oxidized by xanthine oxidase, and that $OH^{\bullet}$ radicals are responsible for this reaction. Acetaldehyde-derived radicals can interact with albumin to form stable adducts. This reaction might be involved in causing acetaldehyde-mediated protein alkylation, possibly contributing to the development of the toxic as well as the immunological reaction ascribed to acetaldehyde.

Acknowledgements: This work was supported by the Ministro dell'Università e della Ricerca Scientifica e Tecnologica (Project Patologia da Radicali Liberi e degli Equilibri Redox) and by the Consiglio Nazionale delle Recerche (Grant 92.02450.CT04) Rome.

References

[1] Lieber, C.S. (1990) Pharmacol. Ther. 46, 1–41.
[2] Nicholls, R., De Jersey, J., Worral, S. Wilce, P. (1992) Int. J. Biochem. 24, 1899–1906.
[3] Mauch, T.J., Donohue, T.M. and Zetterman, K.C. (1986) Hepatol. 6, 263–269.
[4] Sorrell, M.F. and Tuma, D.J. (1987) Ann. NY Acad. Sci. 492, 51–62.
[5] Israel, Y., Hurwitz, E., Niemela, O. and Arnon, R. (1986) Proc. Natl. Acad. Sci. USA 83, 7923–7927.
[6] Koskinas, J., Kenna, J.G., Bird, G.L., Alexander, G.J.M. and Williams, R. (1992) Gastroenterology 103, 1860–1867.
[7] Teare, J.P., Carmichael, A.J., Burnett, F.R. and Reke, M.O. (1993) Alcohol Alcoholism, 28, 11–16.
[8] Nordmann, R., Ribière, C. and Rouach, H. (1992) Free Rad. Biol. Med. 12, 219–240.
[9] Albano, E., Tomasi, A., Goria-Gatti, L. and Dianzini, M.U. (1988) Chem-Biol. Interacts. 65, 223–234.
[10] Albano, E., Tomasi, A., Goria-Gatti, L., Persson, J.O., Terelius, Y., Ingelman-Sundberg, M. and Dianzani, M.U. (1991) Biochem. Pharmacol. 41, 1895–1902.
[11] Shaw, S. and Jayatilleke, E. (1987) Biochem. Biophys. Res. Commun. 143, 984–990.
[12] Shaw, S. and Jayatilleke, E. (1990) Biochem. J. 268, 579–583.
[13] Fridovich, I. (1989) Free Rad. Biol. Med. 7, 557–578.
[14] Puntarulo, S. and Cederbaum, A.I. (1989) Alcohol. Clin. Exp. Res. 13, 84–90.
[15] Albano, E., Parola, M., Comoglio, A. and Dianzani, M.U. (1993) Alcohol and Alcoholism 28, 453–459.
[16] Albano, E., Tomasi, A. and Ingelman-Sundberg, M. (1993) Methods Enzymol. 233, 117–127.
[17] Donouhue, T.M., Tuma, D.J. and Sorrell, M.F. (1983) Arch. Biochem. Biophys. 220, 239–246.
[18] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[19] Nutinen, H., Lindros, K.O. and Salaspuro, M. (1983) Alcohol. Clin. Exp. Res. 7, 163–168.
[20] Kuppusamy, P. and Zweier, J.L. (1989) J. Biol. Chem. 264, 9880–9884.
[21] Lloyd, R.V. and Mason, R.P. (1990) J. Biol. Chem. 265, 16733–16736.
[22] Bounds, P.L. and Winston, G.W. (1991) Free Rad. Biol. Med. 11, 447–453.
[23] Dorfman, L.M. and Adams, G.E. (1973) NSRRS. Natl. Bur. Stand., Washington, DC, 46.
[24] Goria-Gatti, L., Iannone, A., Tomasi, A., Poli, G. and Albano, E. (1992) Carcinogen 13, 799–805.
[25] Battelli, M.G., Abbondanza, A. and Stirpe, F. (1992) Chem.-Biol. Interacts. 83, 73–84.
[26] Shaw, S. and Jayatilleke, E. (1990) Free Rad. Biol. Med. 9, 11–17.
[27] Fridowich, I. (1966) J. Biol. Chem. 241, 3126–3128.
[28] Rajagopalan, K.V. and Handler, P. (1963) J. Biol. Chem. 239, 2027–2035.
[29] Johnson, J.L., Rajagopalan, K.V. and Cohen, H.J. (1974) J. Biol. Chem. 249, 859–866.
[30] Arenson, R.M. (1970) Arch. Biochem. Biophys. 136, 352–360.