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RAPID COMMUNICATIONS

ELECTROCHEMICAL DETERMINATION OF N-OXIDIZED PROCAINAMIDE METABOLITES AND FUNCTIONAL ASSESSMENT OF EFFECTS ON MURINE CELLS IN VITRO

JOHN F. WHEELER, CRAIG E. LUNTE, WILLIAM R. HEINEMAN, LOUIS ADAMS AND EVELYN V. HESS

The Biomedical Chemistry Research Center, Department of Chemistry and College of Medicine, The University of Cincinnati, Cincinnati, OH 45221 and 45267

Because of the implication of N-oxidized metabolites of procainamide in the induction of drug-related lupus, we have studied the electrochemical behavior of these metabolites and developed an electrochemical synthesis of nitrosoprocainamide. This synthesis was developed using procainamide hydroxylamine as the starting material which was oxidized to the nitroso species at an applied potential of 700 mV vs Ag/AgCl using a carbon packed bed bulk electrolysis flow cell. Conversion efficiencies of greater than 95% were achieved with this method. Subsequent studies with a chemically diverse series of biocompounds were used to investigate possible reactions between the procainamide hydroxylamine and nitroso species and these selected molecules. Only antioxidants such as cysteine, glutathione and ascorbic acid were found to react with the nitroso compound as determined by electrochemical methods, and this reaction was characterized as primarily a simple redox reaction at physiological pH. Animal studies conducted with murine spleen cells incubated with mitogens and various procainamide compounds demonstrated that the N-oxidized metabolites are the active immunopharmacologic agents.

INTRODUCTION

The use of the aromatic amine 4-amino-N,N-diethylaminoethylbenzamide, commonly known as procainamide (PA), (Fig. 1) as an anti-arrhythmic drug has been correlated with a high incidence of a positive antinuclear antibody (ANA) test, occasionally with signs and symptoms resembling systemic lupus erythematosus (1). Although drug-related lupus (DRL) has been extensively investigated, the mechanism responsible for the induction of autoimmunity has yet to be elucidated. Much of the PA ingested is acetylated in the liver by acetyl transferase to produce N-acetyl procainamide (NAPA) (Fig. 1) (2). However, recent microsomal studies have indicated that PA hydroxylamine (PAHA) is an N-oxidation product of the cytochrome p-450 mediated metabolism of this drug (2-3). It has been postulated the PAHA metabolite or the further oxidized nitroso metabolite (NOPA) (Fig. 1) may bind irreversibly *in vivo* to some yet to be defined readily available biomolecule, form an antigenic complex, and thus initiate the autoimmune response (2-4). This hypothesis is supported by evidence that synthetically prepared NAPA retains the drug's pharmaceutical action without eliciting the DRL response (5).

A simple and rapid means of generating the potentially reactive NOPA and PAHA metabolites *in vitro* would be useful in investigating reactions with selected biomolecules. The chemical synthesis of PAHA has been reported elsewhere (2-3), however a facile and efficient means of preparing the NOPA species has not been previously described. Following the electrochemical characterization of PA and its oxidized metabolites, an electrolytic synthesis of NOPA from PAHA was developed using a packed carbon bed bulk electrolysis flow cell. Product generation was confirmed to be essentially complete by both cyclic voltammetry (CV) and high-performance liquid chromatography with electrochemical detection (LCEC).

PAHA has been reported to covalently bind to hemoglobin, histone(s) and human serum albumin

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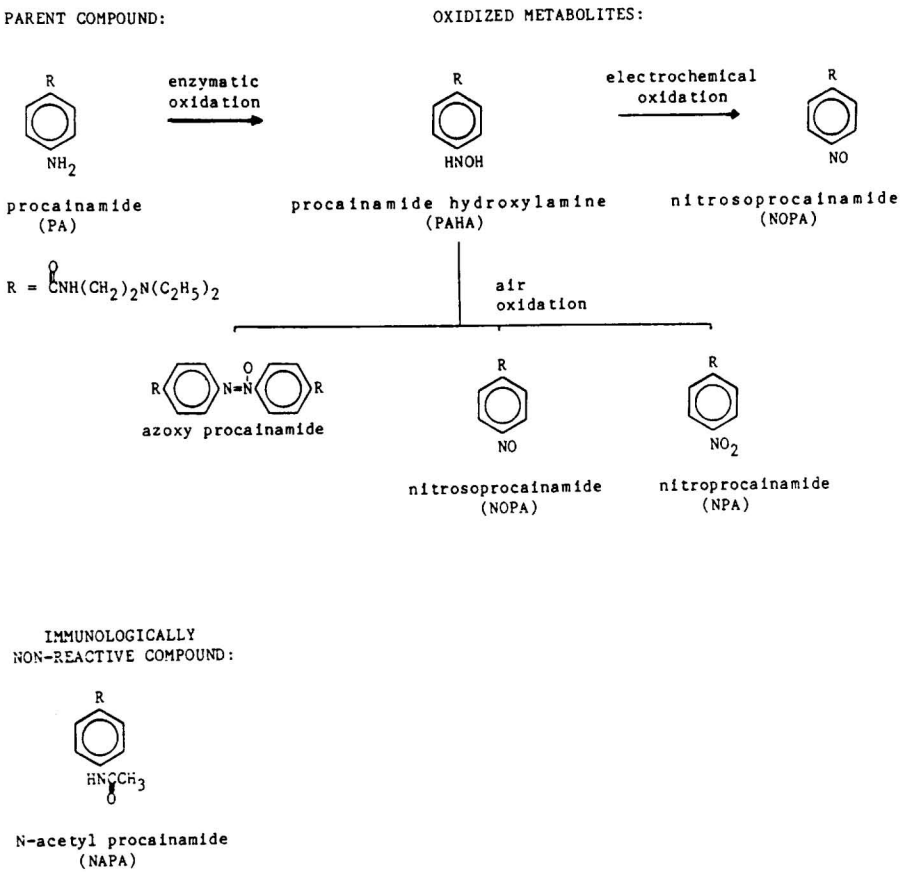

Figure 1: Schematic Diagram of N-acetyl procainamide, procainamide, and N-oxidized procainamide metabolites.

(HSA) in vitro (3-4). Uetrecht has postulated that this interaction may be occurring between the NOPA metabolite and these biomolecules (4). However, direct detection of NOPA has not been previously described. By monitoring current responses using CV and LCEC, the reaction characteristics of PAHA and electrolytically generated NOPA through the derivatized aromatic amine group have been examined with a variety of amino acids, peptides, nucleosides and DNA. Further, we have used PA metabolites to investigate their effects on functional responses of murine cells in vitro.

MATERIALS AND METHODS

Reagents. All chemicals commercially obtained were of reagent grade or better and were used as received. Cysteine, glycine, alanine, methionine, histidine, tyrosine, cystine, guanosine, adenosine, guanine, adenine, thymine, cytidine, glutathione, and PA hydrochloride were obtained from Sigma Chemical (St. Louis, MO). PAHA and nitroprocainamide (NPA) were synthesized as previously reported (3). SS-DNA was prepared in house by the Division of Immunology at University of Cincinnati College of Medicine Culture media, Hank's Balanced Salt Solution (HBSS), RPMI-1640, fetal calf serum (FCS) and antibiotics were obtained from GIBCO (Grand Island, N.Y.).

Apparatus. Cyclic voltammetry was performed with a CV1B voltammetry unit (BAS, W. Laf., IN) with glassy carbon working, platinum auxiliary, and Ag/AgCl reference electrodes. The bulk electrolysis flow cell as modified from the design of Clark and Evans (6) and a potentiostat used for constant applied potential were constructed in the Department of Chemistry at the University of Cincinnati. Similar controlling potentiostats are commercially available. Reticulated vitreous carbon (RVC) and Vycor (thirsty glass) tubing were obtained from BAS. A Sage Model 341 syringe pump (Orion, Cambridge, MA.) was used to provide constant flow. The auxiliary electrode was stainless steel and the reference electrode was Ag/AgCl. The electrolysis cell supporting

electrolyte was 0.2 M potassium nitrate. The LCEC system consisted of a BAS LC154D liquid chromatograph with a glassy carbon dual electrode thin-layer cell and a Ag/AgCl reference electrode. A 5 um ODS RP column (100 x 4.6 mm) (Brownlee, Santa Clara, CA) was used for all separations. A 20 ul sample loop was used. UV/vis absorption spectra were obtained using a Lambda V scanning spectrophotometer (Perkin-Elmer, Norwalk, CT). All potentials reported are vs. Ag/AgCl (3.0 M NaCl).

Cyclic Voltammetry and Bulk Electrolysis Flow Cell Conditions. All CV studies were performed in both a 0.2 M ammonium acetate buffer, pH 5.5, and a 0.2 M THAM buffer, pH 7.4. All voltammograms were recorded at a scan rate of 100 mV/sec. RVC particles for the flow cell were ground to 200 mesh. Flow rates during electrolyses varied between 0.25 and 0.45 mL/min, but were constant within the course of each experiment.

LCEC and Reaction Study Conditions. A flow rate of 1.0 ml/min was used. All samples were prepared in the mobile phase solution, and new standards were prepared fresh weekly in deoxygenated buffer solution and stored at 4° C. Due to varied retention behavior of PA and its oxidized derivatives, two chromatographic conditions were utilized. For the more weakly retained PAHA and PA species, a 0.1 M ammonium acetate buffer, pH 5.5, with 5% acetonitrile (v/v) was used to give retention times of 6.0 and 6.5 min. respectively. For the strongly retained NPA and NOPA species, the acetonitrile concentration was increased to 25% to give retention times of 10.2 and 11.8 min respectively. Binding studies were conducted in both 0.1 M pH 5.5 ammonium acetate and 0.1 M pH 7.4 THAM buffers. All reagent solutions were prepared in buffer and added in 10:1 molar excess to PAHA or NOPA solutions. Incubations were performed for 15 minutes with constant stirring. Samples were deoxygenated prior to analysis. Individual PAHA or NOPA controls were run for each incubation.

Animals. Adult rats (Charles River, Wil. MA) and mice (Bar Harbor, ME) were used in these assays. Briefly, spleens were harvested by standard techniques (7), single cell suspensions prepared, and appropriate cells were pooled and layered onto Ficoll-Hypaque gradients. Recovered cells were washed with HBSS, adjusted to 10^6/ml in RPMI-1640 medium containing 10% FCS and antibiotics and dispersed into microtiter plates (5 x 10^5 cells/well) containing cells alone, varying concentrations of the mitogens or mitogens co-cultured with varying dilutions of the compounds. Cultures were incubated at 37°C with 5% CO_2, pulsed with (^{3}H)-TdR and harvested on a MASH II. DNA synthesis was determined using a scintillation counter.

RESULTS

Bulk Electrolysis. The electrochemical characterization of PA and NPA was achieved by comparison to previously characterized model systems. From this study, it was determined that the best means of generating NOPA would be through the two electron oxidation of PAHA. This oxidation was accomplished using the packed carbon bed flow cell described above at an applied potential of +700 mV vs Ag/AgCl. Due to the electrochemical nature of this technique, isolation of the NOPA response was provided in situ by monitoring current produced from PAHA oxidation. With a high surface area/volume ratio for the RVC working electrode, conversion efficiencies of greater than 95% were afforded using concentrations of PAHA up to 10 mM as determined by LCEC and the derivatized form of Faraday's Law: $i=nFdN/dt$.

LCEC. Dual-electrode LCEC methods were developed for isolation and quantitation of PAHA, NOPA, and NPA. Hydrodynamic voltammetry (HDV) provided additional verification of the ascribed electrochemical behavior of these compounds. HDV's obtained of NOPA reduction and PAHA oxidation indicated current limiting plateau potentials of +450 and -200 mV respectively. An HDV for the four electron reduction of NPA to PAHA indicated a current limiting plateau potential of -650 mV. Consequently, these potentials were used as the detector potentials in all subsequent experiments. While PA could be detected at a potential of 1050 mV, accurate quantitation was prevented by electrode fouling effects.

Qualitative time decay studies of aqueous PAHA indicated multiple product formation with increasingly rapid decomposition in the neutral to basic pH range. PAHA solutions become increasingly yellow as air oxidation occurs over a period of days, indicative of the formation of azoxy-PA, the condensation product of NOPA and PAHA (Fig. 1). Substantial concentrations of both NOPA and NPA were detected based on chromatographic retention with standards, as well as several unidentified components in smaller amounts. Rubin has recently reported the appearance of a UV/vis absorption maxima at 308 nm upon PAHA solution oxidation (8). The UV/vis absorption spectrum of electrolytically generated NOPA indicates a strong absorption at this wavelength not observed for PA, PAHA, or NPA. This provides direct evidence for the <u>in-vitro</u> non-enzymatic oxidation of PAHA to NOPA.

TABLE I

Conversion of Nitrosoprocainamide to Procainamide Hydroxylamine by Selected Reducing Agents

REDUCING AGENT	% CONVERSION TO PAHA[a]	PA DETECTED
Ascorbic Acid, pH 5.5	100	NO
Ascorbic Acid, pH 7.4	100	NO
L-Cysteine, pH 5.5	35	YES
L-Cysteine, pH 7.4	93	NO
Glutathione, pH 5.5	39	YES
Glutathione, pH 7.4	100	NO

a = Values are the mean obtained from 3 separate experiments RSD < 5%.

Reaction Studies. Incubations were performed to detect possible reactions between NOPA and simple biomolecules or PAHA and these molecules. The only amino acid incubation to elicit a measure change in response was cysteine incubated with NOPA. LCEC chromatograms obtained following these incubations demonstrated that the reaction with excess cysteine was complete. PAHA was detected as a major product of this reaction based on chromatographic retention and HDV. Additionally, a thiol/disulfide detection LCEC system constructed by the method of Allison and Shoup (9) indicated the presence of of cystine following these incubations. Ascorbic acid has been suggested to reduce NOPA to PAHA by Rubin (8), and our results confirm this hypothesis, as shown in Table I. Incubations of glutathione (GSH) with NOPA at physiological pH indicate the principle product of this reaction to be PAHA as well (Table I). This is in contrast to previously reported findings of Uetrecht in which PAHA was not detected at pH 6.8 (4).

Antiguanosine antibodies have been reported as a marker for DRL (10). In order to demonstrate whether any direct interactions occur between PAHA or NOPA and nucleosidic molecules, incubations with thymidine, cytidine, guanine, adenine, adenosine and guanosine were performed. No change in monitored current responses was detected by CV or LCEC. Incubations with single-stranded DNA likewise showed no resultant change in these responses, and thus no direct covalent attachment to the oxidized amine functionality.

As shown in Table II, suppression of (^{3}H)-TdR uptake was observed with both PAHA (20 uM) and NOPA (11 uM) incubated with mouse and rat cell cultures for 3 days (Cell viability: PAHA - 45%, NOPA - 60%). Recovery of responses to Concanavalin A (Con A) was observed in both mouse and rat cells cultured with reduced doses of the compounds (Cell viability: 95-97%). Responses with PA and NAPA were not significantly different than control values ($P > 0.05$). Response to Lypopolysaccharide (LPS), however, was significantly reduced from controls with NOPA (1.1 uM), although viability was maintained (86 $\pm$ 3%). In the rat, PAHA produced significant suppression of pokeweed mitogen (PWM) response at both concentrations. This may reflect an affect on T-helper cell function by rat splenic cells.

DISCUSSION

The bulk electrolysis of PAHA to NOPA represents the first synthetic means of efficient NOPA product generation. In addition, CV and LCEC provide an excellent means of selective detection of NOPA. In aqueous solution, NOPA was found to be more long-lived under aerobic conditions than PAHA (2.0% vs 50% decay in 2.0 hr at pH 7.4 THAM), implying that this particular PA metabolite might reach relatively high *in vivo* concentration levels if subsequent binding or redox reactions did not occur.

However, results for the reaction of NOPA with GSH and CYS (Table I) are consistent with the mechanism proposed by Eyer for aromatic nitroso/sulfhydryl interaction (11). In weakly acidic media, the amount of PAHA formed is substantially reduced, and PA is also detected, presumably through hydrolysis of PA-glutathione

Table II.

(^{3}H)-TdR Uptake of Rat and Mouse Splenic Cells Incubated with Optimal Dilution of Mitogens Alone or Co-Cultured with Varying Dilutions of the Compounds.

Species/Strain	Compound	Con A	LPS
Mouse[a] - DBA/2[d]	None	32.0 ± 1.3[b]	21.7 ± 6.3
	NOPA 11 μM	2.2 ± 0.2[c] (93)[d]	3.2 ± 1.3[c] (82)
	NOPA 1.1 μM	29.2 ± 4.0	11.9 ± 1.4[c] (45)
	PAHA 20 μM	1.2 ± 0.8[c] (96)	7.8 ± 0.7[c] (64)
	PAHA 1 μM	29.0 ± 7.0	14.0 ± 1.3
	PA 40 μM	28.1 ± 2.8	17.8 ± 2.4
	PA 4 μM	28.8 ± 2.7	16.8 ± 2.6
	NAPA 36 μM	28.3 ± 2.2	17.7 ± 3.2
	NAPA 3.6 μM	30.4 ± 2.7	19.4 ± 5.1

Species/Strain	Compound	Con A	PWM
Rat[a] - Lewis (RT1)	None	19.0 ± 7.3	16.4 ± 1.0
	NO-PA 11 μM	1.1 ± 0.7[c] (94)	0.2 ± 0.1[c] (99)
	NO-PA 1.1 μM	17.8 ± 3.1	16.5 ± 2.3
	PAHA 20 μM	3.6 ± 2.0[c] (81)	7.9 ± 1.6[c] (52)
	PAHA 1 μM	10.1 ± 5.4	11.1 ± 1.2[c] (32)
	PA 40 μM	18.6 ± 3.6	14.8 ± 2.0
	PA 4 μM	19.2 ± 3.9	15.8 ± 3.0
	NAPA 36 μM	17.9 ± 5.1	14.1 ± 3.6
	NAPA 3.6 μM	18.6 ± 5.7	13.8 ± 2.9

a = Values are the mean ± SD obtained from 4 experiments with 10 mice per experiment or 4 rats per experiment. b = Results expressed as Δ CPM at 10^{-3}. c = The mean ± SD of the experimental group is statistically different than the mean ± SD of the control group ($p < 0.05$). d = Percent suppresion of experimental versus the control value.

sulfinamide. Additionally, PAHA incubated with excess GSH decays much less rapidly under aerobic conditions than PAHA alone (5% vs 50% decay in 2.0 hr). This would suggest that high concentrations of NOPA might not be achieved in vivo in the presence of these reducing species.

Finally, the preliminary animal study results represent the first report of the direct incubation of NOPA on the functional properties of cells. While it remains unclear whether the PAHA or NOPA metabolite may predominate in the cellular matrix, it is apparent that these metabolites are the active immunopharmacologic agents rather than the parent drug itself.

REFERENCES

1. Hess EV. Drug related lupus: The same or different? In: Lahita R, Ed. Systemic Lupus Erythematosus. New York: John Wiley & Sons, p 869-879, 1987.
2. Uetrecht JP, Sweetman BJ, Woosley, RL, Oates JA. Metabolism of procainamide to a hydroxylamine by rat and human hepatic microsomes. Drug Metab Dispos 12:77-81, 1984.
3. Budinsky RA, Roberts, SM, Coats EA, Adams L, Hess, EV. The formation of procainamide hydroxylamine by rat and human liver microsomes. Drug Metab Dispos 15:37-43, 1987.
4. Uetrecht JP. Reactivity and possible significance of hydroxylamine and nitroso metabolites of procainamide. J Pharm Exp Ther 232:420-425, 1985.
5. Lahita R, Kluger J, Drayer DE, Koffler D, Reidenberg MD. Antibodies to nuclear antigens in patients treated with procainamide or acetylprocainamide. N Eng J Med 301:1382- 1385, 1979.
6. Clark BR, Evans DH. Infrared studies of quinone radical anions and dianions generated by flow-cell electrolysis. J Electroanal Chem 69:181-194, 1976.
7. Litwin A, Bash J, Adams LE, Donovan RJ, Hess EV. Immunoregulation of Heymann's nephritis. I. Induction of suppressor cells. J Immun 122:1029-1034, 1979.

8. Rubin RL, Uetrecht JP, Jones JE. Cytotoxicity of oxidative metabolites of procainamide. J Pharm Exp Ther 242:833-841, 1987.
9. Allison LA, Shoup RE. Dual electrode liquid chromatography detector for thiols and disulfides. Anal Chem 55:8-12, 1983.
10. Eyer P. Reactions of nitrosobenzene with reduced glutathione. Chem Biol Int 24:227-239, 1979.
11. Weisbart RH, Wayne SY, Colburn KK, Whang SH, Heng MK, Bouack RJ. Antiguanosine antibodies: A new marker for procainamide-induced systemic lupus erythmatosis. Ann Intern Med 104:310-313, 1986.