Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 437-443

GUANETHIDINE N-OXIDE FORMATION AS A MEASURE OF CELLULAR FLAVIN-CONTAINING MONOOXYGENASE ACTIVITY

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Received March 7, 1983

The usefulness of guanethidine N-oxide formation as a measure of cellular flavin-containing monooxygenase activity was assessed using the purified hog liver enzyme, rat liver microsomes and hepatocytes. The apparent Km and Vmax for this reaction in hepatocytes were 0.30 ± 0.20 mM and 0.81 ± 0.36 nmole per 10^6 cells min^{-1} respectively. The Km for the purified enzyme was 0.31 mM and the Vmax was 0.56 nmole per µg enzyme min^{-1}. 2-Diethylaminoethyl-2,2-diphenyl valerate (SKF-525A) at a concentration of 0.5 mM had no effect on guanethidine N-oxide formation by either rat liver microsomes or the purified enzyme. In contrast 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) at the same concentration caused greater than a 100% increase in the microsomal production of guanethidine N-oxide. The tertiary amines imipramine, chloropromazine and methylpyrilene inhibited N-oxide formation by both hepatocytes and the purified enzyme. These data indicate that guanethidine N-oxide formation can be used as a measure of cellular flavin-containing monooxygenase activity.

Xenobiotics containing nucleophilic nitrogen and sulfur atoms are often substrates for the microsomal and nuclear envelope flavin-containing monooxygenase (1,2). This enzyme was initially referred to as the microsomal amine oxidase but subsequent studies have shown that it catalyzes oxidation of a diverse group of xenobiotics. These include secondary and tertiary amines, hydrazines, disubstituted N-hydroxylamines, thiocarbamides, thioamides, disulfides, sulfides and thiols (1,2). Although this flavoprotein has a broad substrate specificity, certain structural features are necessary before a compound can be metabolized at an appreciable rate (1,2).

The flavin-containing monooxygenase is present in both hepatic and extrahepatic tissues with high activities being found in human and hog

0006-291X/83 $1.50
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liver (3). In hog liver this enzyme can comprise up to 4% of total microsomal protein. However, the exact physiological role of this enzyme is as yet to be established. Ziegler and Poulsen (4) have proposed that its function is to provide oxidizing equivalents to maintain the cellular thiol:disulfide potential. In contrast to the broad substrate specificity of the monooxygenase for exogenous compounds, cysteamine is the only known physiological substrate for this enzyme. This aminothiol is oxidized by the flavin-containing monooxygenase to its disulfide cystamine. The role of this reaction in the reactivation of proteins has been described (6).

The significance of the flavin-containing monooxygenase in thiol:disulfide exchange and in the metabolism of xenobiotics has been based solely on information obtained from subcellular fractions or the purified enzyme. Attempts to study this monooxygenase at the cellular level have been hampered by the instability of its reaction products or competing enzyme pathways for its substrates. Therefore, in order to study this enzyme at the cellular level, there is a need to establish a reliable and sensitive method for determining its activity. The tertiary amine guanethidine has previously been shown to be a substrate of the flavin-containing monooxygenase and forms a relatively stable N-oxide (1). In the present study we report the development of a sensitive radiometric high performance liquid chromatographic method to measure the N-oxide of guanethidine, and propose that the N-oxidation of this tertiary amine can be used as a probe for the expression of flavin-containing monooxygenase activity in isolated cell preparations.

METHODS

Enzyme and Chemicals: Purified hog liver flavin-containing monooxygenase enzyme was generously donated by Dr. D. M. Ziegler, The University of Texas at Austin, Texas. Guanethidine sulfate and 2,4,dichloro-6-phenylphenoxyethylamine (DPEA) were kind gifts of Ciba Pharmaceutical Company, Summit, NJ, and Lilly Research Laboratories, IN, respectively. [^{14}C]-Guanethidine and [^{14}C]-guanethidine N-oxide were synthesized according to the method of Abramson et al (6). NADPH was obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals were of the highest purity commercially available.

Animals and Preparation of Hepatocytes and Microsomes:

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 200-250 g, were provided with food and water ad libitum. Hepatocytes were prepared according to the method of Williams et al (7). Cells were resuspended and incubated in Williams Medium E (pH 7.4 at 37°C) containing 5.5 mM glucose and 25 mM Hepes. Cell viability as judged by trypan blue exclusion was at least 86%. Half a milliliter of the cell suspension containing 1 x 10^6 viable cells was incubated with 2 mM [^{14}C]-guanethidine (3.4 mCi/mmole) at 37°C in a shaking water bath under an atmosphere of 95% oxygen/5% carbon dioxide. The blank consisted of hepatocytes treated with acetonitrile, at zero time, and then incubated with [^{14}C]-guanethidine. Microsomes were prepared as previously described (8). A standard 0.40 ml incubation mixture contained microsomal protein 1.5 mg, phosphate buffer (pH 7.4) 83 mM, NADPH 1.8 mM, and 2 mM [^{14}C]-guanethidine. Conditions for the purified hog liver flavin-containing monooxygenase enzyme were as above except a glycine buffer (pH 8.4) was used and microsomal protein was replaced with 25 µg of the purified enzyme. NADPH was omitted from the blanks and replaced by an equal volume of buffer. All reactions were commenced by the addition of substrate and carried out in air at 37° in a metabolic shaker for 10 minutes. Reactions were stopped by the addition of 0.40 ml of acetonitrile and spun at 2500 g for 10 minutes to precipitate the protein. A 150 µl aliquot was taken for analysis by high performance liquid chromatography.

Chromatography: Separation of the N-oxide of guanethidine was achieved using a Radial Compression Module 100 equipped with a Radial-Pak CN cartridge (10 µM, 5mM x 10 CM) and eluted isocratically with a 27% acetonitrile:H_2O solvent containing 0.02 M dibutylamine, pH 7.8. The flow rate was 1 ml/min for 5 minutes followed by 3 ml/min for 15 minutes. Fractions were collected every 30 seconds and radioactivity was measured, after the addition of 12 ml of Aquasol, in a Beckman LS 9000 liquid scintillation spectrometer equipped with automatic external standardization.

Mass Spectrometry: Spectra of guanethidine sulfate and its N-oxide sulfate were recorded using a V-G Micromass 7070E mass spectrometer equipped with a Fast Atom Bombardment (FAB) ionization source and a Xenon atom gun. Samples were loaded on the probe as glycerol emulsions and the spectra were collected in the positive ion mode. The low and high resolution electron impact spectra on the hexafluoroacetylacetone derivatives of guanethidine (9) and of its N-oxide were performed using a JEOL made JMS-01SG-2 mass spectrometer equipped with a solid sample inlet mode and photoplate detection capability.

RESULTS AND DISCUSSION

Mass spectral measurements provided unambiguous proof that the chemically synthesized guanethidine N-oxide, which was used for direct chromatographic comparisons of the biologically derived compound, possessed the assigned structure. For example, the pseudomolecular ion (MH^+, M/Z 215) was found to be the base peak in the FAB spectrum of the N-oxide, demonstrating the presence of one oxygen atom in the molecule. The location of the oxygen atom on the tertiary heterocyclic nitrogen was confirmed by the presence

of an ion at M/Z 142 in the electron impact spectrum of the hexafluoroacetyl-acetone derivative of guanethidine N-oxide. This ion, with elemental composition of $C_8H_{16}NO$ corresponds to a N-oxyfomaldiminium ion containing the heptamethyleneimine end of the molecule.

Guanethidine N-oxide production was linear up to 160 µg of purified hog liver enzyme per milliliter and over a time period of 10 minutes. When the concentration of guanethidine was varied from 0.04-4.0 mM a Km and Vmax for guanethidine N-oxide production of 0.31 ± 0.01 mM and 0.56 ± 0.03 nmol μg^{-1} enzyme min^{-1} were obtained, respectively. Similarly, the linearity of this reaction with time and cell number was studied in freshly isolated rat hepatocytes (Fig. 1a & 1b). Guanethidine N-oxide formation was linear up to 2 x 10^6 cells per milliliter and over a time period of 20 minutes. The apparent Km and Vmax values for this reaction in hepatocytes were 0.30 ± 0.20 mM and 0.81 ± 0.36 nmol per 10^6 cells min^{-1} (mean ± SEM, n = 3), respectively (Fig. 2). The Km's reported in this study are similar to a Km of 0.17 mM given by Ziegler (1) for this reaction using the purified hog liver enzyme.

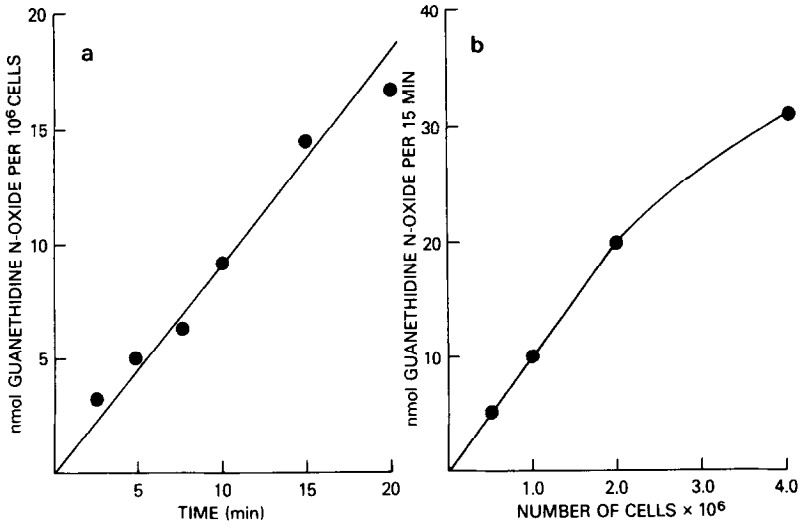

Fig. 1. Guanethidine N-oxide formation by rat hepatocytes as a function of time (a) and cell number (b). Conditions were the same as under methods except the incubation volume was 1 ml.

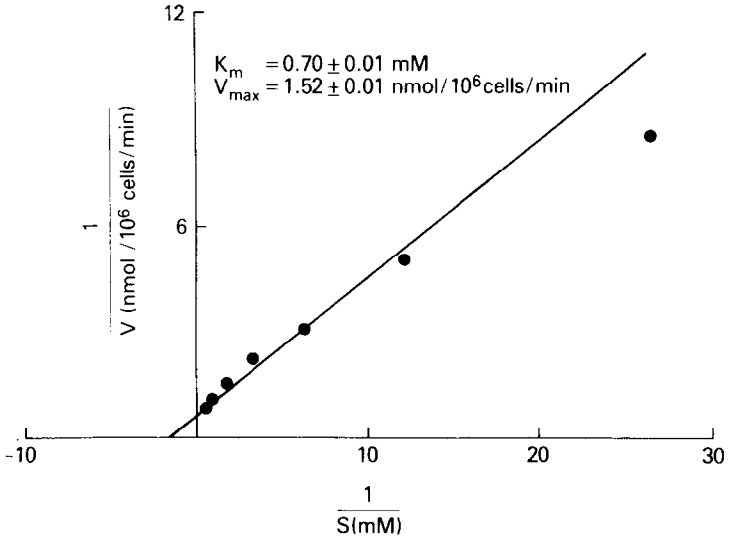

Fig. 2. Lineweaver-Burk plot of guanethidine N-oxidation in rat hepatocytes. The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) were determined using the method of Wilkinson (12).

The capacity of tertiary amines that are known substrates of the flavin-containing monooxygenase to inhibit guanethidine N-oxide formation in hepatocytes and by the purified enzyme were assessed. Concentrations used to inhibit the enzyme activity in hepatocytes were three times the Km's for the N-oxidation of these amines (1). Table 1 shows that imipramine, chlorpromazine and methapyrilene all substantially inhibited

Table 1. Effect of Tertiary Amines on Rat Hepatocyte Guanethidine N-Oxide Formation

Addition	Concentration (μM)	nmol guanethidine N-oxide per 10^6 cells min^{-1}	Percent of Control
Control	-	0.32 ± 0.01	100
Imipramine	66	0.17 ± 0.02[a]	53
Chlorpromazine	27	0.21 ± 0.02[a]	66
Methapyrilene	279	0.13 ± 0.01[a]	41

Values represent mean ± SEM, n = 4

[a] Significantly different from control ($p < 0.05$)

hepatocyte N-oxide formation. The concentration of these compounds to inhibit the activity of the purified enzyme by a similar amount were 1.6-7.4 times higher than those used to inhibit hepatocyte N-oxidation (Table 2).

Methods used to differentiate between flavin-containing and cytochrome P_{450} monooxygenase activities have generally employed inhibitors of cytochrome P_{450} (10). SKF-525A has been classically used to distinguish between cytochrome P_{450} and flavin-containing monooxygenases, and when no effect is observed with this inhibitor the reaction has been assumed to be mediated by the latter enzyme. In the present study SKF-525A had no effect on guanethidine N-oxide formation either in microsomes or by the purified enzyme (Tables 2 and 3). In addition, metyrapone and α-naphthoflavone, selective inhibitors for the phenobarbital and methylcholanthrene induced forms of P_{450}, respectively (11), caused no decrease in guanethidine N-oxide formation. The effect of metyrapone was to increase enzyme activity and this was concentration dependent. Another indication that guanethidine N-oxide formation is mediated by the flavin-containing monooxygenase in microsomes is that DPEA stimulates this reaction (Table 3). Primary amines such as DPEA have been shown to bind to the effector site of the enzyme and double the catalytic activity of the enzyme (1).

These data clearly show that the flavin-containing monooxygenase mediates the N-oxidation of guanethidine, and that the N-oxide formed can

Table 2. Effect of Tertiary Amines on Guanethidine N-Oxide Formation by the Purified Hog Liver Flavin-Containing Monooxygenase

Addition	Concentration (μM)	nmol guanethidine N-oxide μg^{-1} enzyme min^{-1}	Percent of Control
Control	-	0.54 ± 0.01	100
Imipramine	200	0.33 ± 0.02[a]	61
Chlorpromazine	200	0.41 ± 0.02[a]	76
Methapyrilene	465	0.25 ± 0.01[a]	46
SKF-525A	500	0.49 ± 0.01[a]	91

Values represent mean ± SEM, N = 3

[a] Significantly different from control ($p < 0.05$)

Table 3. Effect of Cytochrome P_{450} Inhibitors on Rat Liver Microsomal Guanethidine N-Oxide Formation

Addition	Concentration (mM)	nmol guanethidine N-oxide mg protein^{-1} min^{-1}
Buffer control	--	0.64 ± 0.07
SKF-525A	1.0	0.76 ± 0.07
	2.0	0.52 ± 0.05
DPEA	0.25	1.34 ± 0.21[a]
	1.0	1.41 ± 0.15[a]
Ethanol Control[b]	--	0.44 ± 0.09
Metyrapone	0.5	0.89 ± 0.15
	2.0	1.03 ± 0.16[a]
α-naphtho-flavone	0.25	0.49 ± 0.15
	0.50	0.53 ± 0.20

Values represent mean ± SEM, n = 3.

[a] Significantly different from control ($P < 0.05$)

[b] Metyrapone and α-naphthoflavone were added to the incubation media in 10 µl ethanol.

be measured in isolated rat hepatocytes. Further, N-oxidation of guanethidine, based on inhibition data, is mediated solely by the flavin-containing monooxygenase.

ACKNOWLEDGMENTS

We thank Dr. J. A. Kelley of the National Cancer Institute for performing the FAB mass spectral measurements, and Dr. D. M. Ziegler, University of Texas, for his helpful advice.

REFERENCES

1. Ziegler, D. M. (1980) Enzymatic basis of detoxication, 201-227, Academic Press, New York.
2. Poulsen, L. L. (1981) Rev. Biochem. Toxicol. 3, 33-49.
3. Poulsen, L. L. and Ziegler, D. M. (1979) J. Biol. Chem. 254, 6449-6455.
4. Ziegler, D. M. and Poulsen, L. L. (1977) Trends Biochem. Sci. 2, 79-81.
5. Poulsen, L. L. and Ziegler, D. M. (1977) Arch. Biochem. Biophys. 183, 563-570.
6. Abramson, F. B., Furst, C. I., McMartin, C. and Wade, R. (1969) Biochem. J. 113, 143-156.
7. Williams, G. M., Bermudez, E. and Scaramazzino, D. (1977) In Vitro 13, 809-817.
8. McManus, M. E., Lang, M. A., Stuart, K. and Strong, J. (1982) Biochem. Pharmacol. 31, 547-552.
9. Erdtmansky, P. and Goehl, T. J. (1975) Anal. Chem. 47, 750-752.
10. Ulhleke, H. (1971) Xenobiotica 1, 327-338.
11. Levine, W. G. and Lu, L. Y. H. (1982) Drug Metab. Dispos. 10, 102-109.
12. Wilkinson, G. N. (1961) Biochem. J. 80, 324-332.