Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 323-327

INHIBITION OF CYTOCHROME C OXIDASE BY PSYCHOSINE (GALACTOSYLSPHINGOSINE)

Hideki Igisu[1] and Michio Nakamura[2]

[1]Department of Environmental Toxicology, University of Occupational and Environmental Health, Kitakyushu 807, Japan

[2]Department of Internal Medicine, Koga Hospital, Kurume 830, Japan

Received April 17, 1986

Pi uptake and acetoacetate formation were suppressed by psychosine (galactosylsphingosine) in rat liver mitochondria. Besides, reduced form of cytochrome c increased in the reaction mixture which contained psychosine. Using reduced form of cytochrome c as substrate, less than 5 μM of psychosine (0.1 μmoles/mg of mitochondrial protein) inhibited cytochrome c oxidase by more than 50%. The inhibition was completely reversed by 1% human serum albumin. Thus, a lipid which is produced in the brain has a powerful and yet reversible inhibitory effect on an enzyme of cellular respiration.

Psychosine (galacotsylsphingosine) is enzymatically synthesized in the brain from UDP-galactose and sphingosine [1], and hydrolized by galactosylceramidase [2]. Probably because of the rapid turnover, psychosine is not detectable in normal brain [3] but when the degradating enzyme is missing, as in Krabbe disease (globoid cell leukodystrophy)[4], psychosine accumulates in the brain (in man, dog and mouse)[3,5]. This accumulation of psychosine is considered the basis of the devastating pathology in Krabbe disease [3] since psychosine is cytotoxic [6]. However, the mechanism of the cytotoxicity has not been clarified. We found that psychosine inhibits cytochrome c oxidase.

Materials and Methods

Psychosine was purchased from Sigma, St Louis, MO, cytochrme c, ADP, NAD and hexokinase from Boehringer-Mannheim-Yamanouchi, Tokyo, and human serum albumin from Chemical Dynamic Corp, South Plainfield, NJ. All other chemicals were reagent grade.

Reduced form of cytochrome c was prepared by adding a small amount of sodium hydrosulfite (approximately 0.1%) to 1% cytochrome c dissolved in 10 mM Tris-HCl pH 7.4 containing 0.1 mM EDTA-2K and dialized against the same buffer (without sodium hydrosulfite) for 20 hrs with three changes of the solution.

0006-291X/86 $1.50

Mitochondria were prepared following Borgese and Meldolesi [7] with some modifications. Male Wistar rats weighing 200-300g were killed by decapitation. Liver was removed rapidly, rinsed in 0.25 M sucrose containing 5 mM Tirs-HCl pH7.4, minced and homogenized in a Potter-Elvehjem homogenizer with a loose teflon pestle (700 rpm, 10 strokes) in 4 volumes of 0.25 M sucrose containing 5 mM Tris-HCl pH7.4. After nuclei and cell debri were removed by centrifugation at 500 g_{AVE} for 10 min, the supernate was centrifuged at 8100 g_{AVE} for 10 min. The crude mitochondrial pellet was suspended in the same solution (5 ml/g equivalent of liver) and sedimented by the same centrifugation. The pellet was re-suspended in 0.4 M sucrose containing 5 mM Tris-HCl pH7.4 and centrifuged at 4500 g_{AVE} for 10 min. All procedures were done at 4°. The mitochondria were suspended in 0.25 M sucrose containing 5 mM Tris-HCl pH7.4 and the protein was determined by the method of Lowry et al [8].

Pi uptake and acetoacetate formation were examined using β-hydroxybutyrate as substrate [9]. The reaction mixture [10] (1.0 ml) contained 40 mM glycylglycine buffer pH7.4, 25 mM DL- β -hydroxybutyrate, 10 mM potassium-phosphate buffer pH7.4, 2.5 mM ADP, 1.2 mM NAD, 25 mM glucose, 0.4 mg hexokinase, 5 mM $MgCl_2$, 15 mM NaF and 0.019% cytochrome c (oxidized form). Psychosine was dissolved in 4 mM glycylglycine buffer using water-bath type sonicator and added to the reaction mixture. The reaction was started by adding mitochondrial suspension (0.1 ml) to the reaction mixture. After incubation for 15 min at 30°, the reaction was terminated by adding 0.1 ml of 25% ice-cold perchloric acid. After centrifugation at 1000 g for 15 min, acetoacetate [11] and Pi [12] in the supernate were measured.

To obtain the absorbance spectrum of the reaction mixture containing psychosine, equal amount of the reaction mixture (0.85 ml) was put in "sample" and "reference" cuvette in a Hitachi 557 spectrophotometer. After the base line was corrected by the computer, 0.15 ml of 5 mM Tris-HCl pH7.4 and 0.15 ml of 1 mM psychosine dissolved in the same buffer was added to the reference and the sample cuvette, respectively, and the spectrum was recorded.

NADH-cytochrome c reductase, succinate-cytochrome c reductase and cytochrome c oxidase were determined measuring the changes of the absorbance of (550-540)nm using a Hitachi spectrophotometer (dual wave length mode). The slit of the light source was 1 nm. The temperature of the cuvette holder was kept at 25° with a Lauda RM3 circulating water bath. The reaction mixture (0.8 ml) for the assay of these enzymes were as described [13,14] except that 50 mM glycylglycine (instead of phosphate) buffer pH7.4 and 2 mM EDTA-2K were used and that it contained 3.1 mM Tris-HCl pH7.4 because psychosine was first dissolved in 5 mM Tris-HCl pH7.4. After the temperature of the reaction mixture was equilibriated at 25° for 2.5 min, 20 µl of mitochondrial suspension (40 µg of protein) was added and the reaction was started.

Results and Discussion

Both Pi uptake and acetoacetate formation in mitochondria were inhibited by psychosine (Fig. 1). Besides, the color of the reaction mixture to which psychosine was added turned to lightly red soon after the incubation was started. The differential spectrum of the reaction

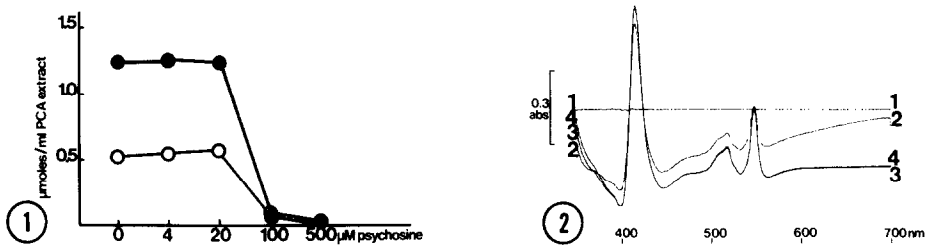

Fig. 1 Effects of psychosine on Pi uptake (●) and acetoacetate formation (○). The protein content of the mitochondrial suspension (0.1 ml) was 1.7 mg. The constituent of the reaction mixture (1.0 ml) is described in Materials and Methods.

Fig. 2 Differential spectrum of the absorbance of the reaction mixture with and without psychosine. Equal amount (0.85 ml) of the reaction mixture (mitochondrial protein 0.9 mg) was put in the sample and the reference cuvette and the base line was corrected by the computer (1). Then, equal amount (0.15 ml) of 5 mM Tris-HCl pH7.4 and 1 mM psychosine in the same buffer was added to the reference and the sample cuvette, respectively. The absorbance spectrum scan (120 nm/min) was started immediately after the addition of psychosine (2), 5 min (3) and 10 min later (4).

mixture with and without psychosine clearly indicated the increase of reduced form of cytochrome c in the mixture which contained psychosine (Fig. 2). These led us to suspect that psychosine might inhibit cytochrome c oxidase.

Indeed, psychosine showed a powerful inhibitory effect on cytochrome c oxidase. The effect was immediately apparent on starting the reaction and less than 5 μM of psychosine (0.1 μmoles/mg protein of mitochondria) suppressed the activity by more than 50% (Fig. 3). Psychosine also inhibited NADH-cytochrome c reductase but the effect was much weaker (less than 1/10). Succinate-cytochrome c reductase was not inhibited but appeared activated by psychosine of 50 μM or less.

On the other hand, when human serum albumin was added (final concentration of 1%), the inhibition of cytochrome c oxidase by psychosine was completely reversed (Fig. 4). This shows that suppression of cytochrome c oxidase by psychosine was not caused by non-specific denaturation of the enzyme. This also suggests that the degree of

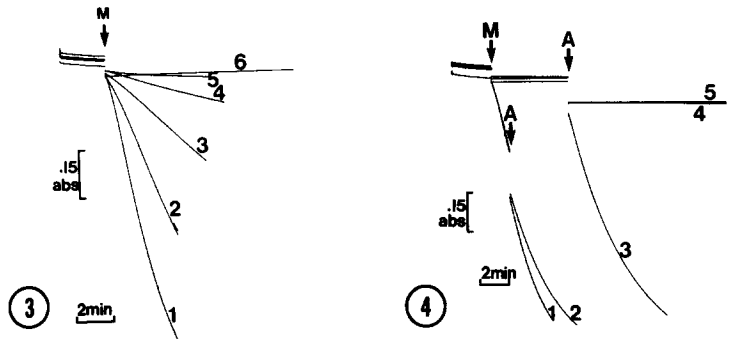

Fig. 3 Effects of psychosine on cytochrome c oxidase. The speed of the decrease of the absorbance (550-540 nm) represented the activity of the enzyme. The reaction mixture (0.8 ml) contained 50 mM glycylglycine buffer pH7.4, 2 mM EDTA-2K, 0.08% cytochrome c (reduced form), 3.1 mM Tris-HCl pH7.4, and appropriate amount of psychosine. Psychosine was dissolved in 5 mM Tris-HCl pH7.4 and added to the reaction mixture. Twenty µl of mitochondrial suspension (40 µg of protein) was added (at M in the figure) and the reaction started. The final concentration of psychosine was 0 (1), 1.56 (2), 6.25 (3), 25 (4), 50 (5) and 100 µM (6).

Fig. 4 Effects of human serum albumin. Experimental conditions were same as in Fig. 3 except that 80 µl of 10% human serum albumin (dissolved in water) or water was added at A. Albumin and water was added to (1) and (2), respectively. No psychosine was present in either (1) or (2). Cytochrome c oxidase was completely inhibited for 5 min by 100 µM psychosine in (3) until albumin was added and the enzyme activity was completely restored. On the other hand, no enzymatic activity appeared when water was added to (4) which contained 100 µM psychosine. No mitochondria were present (20 µl of 0.25 M sucrose containing 5 mM Tirs-HCl pH7.4 was added at M) in (5) and albumin was added.

the cytotoxicity of psychosine may differ among different cells; cells containing higher protein may be more resistent to psychosine.

At present, the physiological role of psychosine is unclear and there is no evidence that psychosine exerts the cytotoxicity by inhibiting cytochrome c oxidase *in vivo* (in Krabbe disease or possibly in other conditions). But it seems noteworthy that a lipid, which is synthesized in the brain, has a powerful inhibitory effect on an enzyme of cellular respiration.

Acknowledgements

We thank Prof. S. Minakami (Department of Biochemistry, Faculty of Medicine, Kyushu University) for valuable comments and Prof. N. Inoue (Department of Environmental Toxicology, University of Occupational and Environmental Health) for encouragement.

References

1. Cleland, W.W., and Kennedy, E.P. (1960) J. Biol. Chem. 235, 45-51.
2. Miyatake, T., and Suzuki, K. (1972) J. Biol. Chem. 247, 5398-5403.
3. Igisu, H., and Suzuki, K. (1984) Science 224, 753-755.
4. Suzuki, K., and Suzuki, Y. (1970) Proc. Natl. Acad. Sci. USA 66, 302-309.
5. Svennerholm, L., Vanier, M.T., and Mansson, J.E. (1980) J. Lipid Res. 21, 53-64.
6. Taketomi, T., and Nishimura, K. (1964) Japan. J. Exp. Med. 34-5, 255-265.
7. Borgese, N., and Meldolesi, J. (1980) J. Cell Biol. 85, 501-515.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Lehninger, A.L., and Smith, S.W. (1949) J. Biol. Chem. 181, 415-429.
10. Weinbach, E.C., and Garbus, J. (1956) Nature 178, 1225-1226.
11. Walker, P.G. (1954) Biochem. J. 58, 699-704.
12. Taussky, H.H., and Shorr, E. (1953) J. Biol. Chem. 202, 675-685.
13. King, T.E. (1967) Meth. Enzym. 10, 216-222.
14. Coruzzi, G., Trembath, M.K., and Tzagoloff, A. (1979) Meth. Enzym. 56, 95-106.