Radiorespirometric Analysis of Glucose Metabolism in the Rat during Feeding with 3'-Methyl-4-(dimethylamino) azobenzene

—Radiorespirometry using $[1-^{14}\text{C}]$ glucose—

Shyuji KOJIMA, Nagatoshi ISHII*, Yasuko SHIKI* and Akiko KUBODERA*

Faculty of Pharmaceutical Sciences, Teikyo University, Suarashi 1091-1, Sagamiko-machi,

Tukui-gun, Kanagawa 199-01, *Faculty of Pharmaceutical Sciences, Science University

of Tokyo, Funagawara-cho 12, Ichigaya, Shinjuku-ku, Tokyo 162

Received April 4, 1979

We estimated the enzyme activities on the hexose monophosphate oxidative pathway of glucose metabolism at an early stage during feeding with 3'-methyl-4-(dimethylamino) azobenzene (3'-Me-DAB) in the first paper of this series. In the present paper, the importance of this non-glycolytic pathway of glucose metabolism in preneoplastic liver in azo-dye fed rat was studied by radiorespirometry using $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose. In addition to radioisotope analysis, the level of activity of glucose-6-phosphate dehydrogenase was measured in rat liver after various periods of feeding with 3'-Me-DAB.

In radiorespirometry using $[1-^{14}\text{C}]$ glucose, the peak time was delayed up to the 2nd week, but was early by the 4th week and was the same as the control at the 5th week and thereafter. On the other hand, the peak height and the yield value both changed in almost the same way. That is to say, they were lower than those of the control until the 2nd week, began to increase from the 3rd week, reached a maximum at the 4th week, and remained at the control level at the 5th week and thereafter. Moreover, the yield value ratio of respiratory $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glucose to that from $[6-^{14}\text{C}]$ glucose increased from 1.3 to 1.52 at the 3rd to 4th week after feeding with 3'-Me-DAB.

The activity of glucose-6-phosphate dehydrogenase increased as early as the 2nd week after the start of feeding with 3'-Me-DAB, reaching a maximum at the 4th week. Thereafter, the activity remained high with a slight decrease from the maximum by the 6th week.

The incorporations of $[^3\text{H}]$ thymidine and $[U-^{14}\text{C}]$ glucose into rat liver DNA at the 4th week after feeding with 3'-Me-DAB were both as high as 2 to 4 times those of the control.

Key Words: 3'-methyl-4-(dimethylamino) azobenzene, $[1-^{14}\text{C}]$ glucose, $[6-^{14}\text{C}]$ glucose, hexose monophosphate oxidative pathway, radiorespirometry

1. Introduction

In our previous paper¹⁾, we suggested that the hexose monophosphate (HMP) oxidative pathway was activated at an early stage during feeding with 3'-methyl-4-(dimethylamino) azobenzene (3'-Me-DAB), on the basis of the changes in radiorespirometry and glycolytic enzyme activity.

It is well known that the HMP pathway is active in cases of tumors²⁻¹²⁾, liver injury¹³⁾, regenerating liver¹⁴⁾, embryos, and hyperthyroid-

ism¹⁵⁻¹⁶⁾, of experimental animals. Since one of the main functions of this pathway is to supply ribose-5-phosphate for incorporation into nucleic acids and coenzymes, it is expected to play an important role in glucose metabolism in tumors and other actively dividing cells.

Watabe¹⁷⁾ reported that a transient appearance of serum α -fetoprotein at an early stage of azo-dye carcinogenesis in the rat largely coincided with the time of rapid oval cell proliferation. Thus, our previous report¹⁾ could be related to oval cell proliferation at an early stage during

feeding with 3'-Me-DAB.

In this paper, radiorespirometry using $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose was carried out in order to confirm the activation of the HMP pathway at an early stage during feeding with 3'-Me-DAB, and the activity of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP⁺ oxidoreductase, EC 1.1.1.49), was also determined.

2. Materials and Methods

Male rats of the Donryu strain (Nihon Rat Co., Urawa), weighing about 180 g were fed on a synthetic diet containing 0.06% of 3'-Me-DAB (Oriental Yeast Co. Ltd., Tokyo, Japan) for 6 weeks. The control rats were fed on the basal diet, CE-2 (Clea Japan Inc., Tokyo), throughout the experiments.

2.1 Radiorespirometry

The radiorespirometric patterns were obtained by the method¹⁾ described previously, using $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose as substrates. The labelled glucose substrates were purchased from New England Nuclear Co., USA. The specific activity of $[1-^{14}\text{C}]$ glucose was 57 mCi/mmole, and that of $[6-^{14}\text{C}]$ glucose was 53 mCi/mmole. In each experiment, 2.5 μCi of a labelled glucose was injected intraperitoneally every week for 6 weeks during feeding with 3'-Me-DAB.

2.2 Assays of pyruvate kinase and glucose-6-phosphate dehydrogenase

Activity of pyruvate kinase (PK) was measured as described previously¹⁾. Glucose-6-phosphate dehydrogenase (G-6-PD) was determined spectrophotometrically at 37° by the rate of NADPH formation at 340 nm with a Gilford recording spectrophotometer 250. The reaction mixture consisted of 2.0 ml of 0.25 M glycylglycine (pH 7.6), 0.5 ml of 0.1 M MgCl_2 , 0.05 ml of liver supernatant and 0.05 ml of NADP (10 mg/ml). The reaction was started by the addition of 0.1 ml of 0.05 M glucose-6-phosphate to both cells, the blank being devoid of NADP. Supernatant fractions from 50% (w/v) liver homogenates in 0.15 M KCl containing 1 mM EDTA, 0.02 M mercaptoethanol, and 0.005 M MgCl_2

(pH 7.4) were prepared by centrifugation at 30,000 g for 60 minutes at 0°. The activity was expressed in standard units (μmoles of NADPH formed per minute). Protein contents were determined by the method of Lowry, et al¹⁸⁾.

2.3 Incorporations of $[^3\text{H}]$ thymidine and $[\text{U-}^{14}\text{C}]$ glucose into rat liver DNA

At the 4th week after feeding with 3'-Me-DAB, rats were injected intraperitoneally with 40 μCi of $[\text{U-}^{14}\text{C}]$ glucose (specific activity: 333 mCi/mmole), and 40 μCi of $[^3\text{H}]$ thymidine (specific activity: 48 Ci/mmole), both New England Nuclear Co., USA and sacrificed 2 hr later. The livers were removed after rapid perfusion with isotonic saline, then blotted, and weighed. One gram of liver was homogenized in 5 volumes of cold 0.03 M Tris-HCl buffer (pH 7.4) using a Potter-type glass homogenizer. The nucleic acid was fractionated from the homogenate by the methods of Schmidt and Thannhauser¹⁹⁾ and of Schneider²⁰⁾. Radioactivities of ^{14}C and ^3H in DNA extracts were measured in toluene-triton X-100 (2:1) scintillator in an Aloka LSC-651 liquid scintillation counter with external standardization.

3. Results

3.1 Radiorespirometry

The contribution of HMP pathway to glucose metabolism at an early stage during feeding with

Fig. 1 Changes in peak time during feeding with 3'-Me-DAB. Results were calculated as percent of the control. The control was arbitrarily taken as 100%. Vertical lines indicate the standard deviations ($n=4$).

Fig. 2 Peak heights during feeding with 3'-Me-DAB. Results were calculated as percent of the control. The control was arbitrarily taken as 100%. Vertical lines indicate the standard deviations ($n=4$).

Fig. 3 Yield values during feeding with 3'-Me-DAB. Results were calculated as percent of the control. The control was arbitrarily taken as 100%. Vertical lines indicate the standard deviations ($n=4$).

3'-Me-DAB was estimated from the changes in radiorespirometry and in glycolytic and non-glycolytic enzyme activities. In order to assess the relative importance of the non-glycolytic pathway of carbohydrate metabolism, we carried out radiorespirometry using $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose for 6 weeks during feeding with 3'-Me-DAB. The results are shown in Figs. 1-4. As shown in Fig. 1, peak time (PT) was markedly delayed at the 1st week, and recovered from the 2nd to reach the control level at the 3rd week. At the 4th week, however, PT was

Fig. 4 Yield value ratios of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glucose to that from $[6-^{14}\text{C}]$ glucose during feeding with 3'-Me-DAB. Values represent the mean $\pm$ standard deviation ($n=4$).

Fig. 5 Activities of G-6-PD and PK during feeding with 3'-Me-DAB. Results were calculated as percent of the control. The control was arbitrarily taken as 100%. Vertical lines indicate the standard deviations ($n=4$).

early, but by the 5th week and thereafter, it coincided with the control. The peak height (PH), as shown in Fig. 2, decreased to about 70% of the control level at 1st to 2nd week

but started to increase at the 3rd week, reaching a maximum at 4th week. The yield value (YV) (Fig. 3) showed almost the same pattern of changes as the peak height. On the other hand, the pattern of the yield value ratio of respiratory $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]$ glucose to that from $[6\text{-}^{14}\text{C}]$ glucose is shown in Fig. 4. The ratio increased from 1.3 to 1.52 at the 3rd to 4th week during feeding with 3'-Me-DAB.

3.2 Activities of G-6-PD and PK

The changes of G-6-PD activity are shown in Fig. 5. The activity began to rise significantly from the 2nd week, reaching about 5 times the control level, and thereafter the activity remained high, with little decrease from the maximum by the 6th week. In addition, PK, a key enzyme in the glycolytic pathway, also as shown in Fig. 5, was markedly inhibited immediately after the start of feeding with 3'-Me-DAB and recovered to the control level by the 5th week.

3.3 $[^3\text{H}]$ thymidine and $[\text{U}\text{-}^{14}\text{C}]$ glucose incorporations into rat liver DNA

The incorporations of $[^3\text{H}]$ thymidine and $[\text{U}\text{-}^{14}\text{C}]$ glucose into rat liver DNA at the 4th week after the administration of 3'-Me-DAB are summarized in Table 1. In the 4th week, they increased by 180% for $[^3\text{H}]$ thymidine and 390% for $[\text{U}\text{-}^{14}\text{C}]$ glucose respectively, over the control.

Table 1 Incorporations of $[^3\text{H}]$ thymidine and $[\text{U}\text{-}^{14}\text{C}]$ glucose into rat liver DNA

	$[^3\text{H}]$ thymidine (dpm/mg DNA)	$[\text{U}\text{-}^{14}\text{C}]$ glucose (dpm/mg DNA)
Control	18,335 $\pm$ 3,667 (100)	5,520 $\pm$ 1,741 (100)
3'-Me-DAB	72,779 $\pm$ 10,917 (397)	9,983 $\pm$ 2,445 (181)

* Three rats were used for each group. They were received 40 μCi of each labelled compound at 4th week after the start of feeding with 3'-Me-DAB. Each value represents the mean $\pm$ standard deviation. Figures in parentheses are percentages relative to the control (100%).

4. Discussion

In order to determine whether the non-glycolytic pathway of glucose metabolism is

activated at an early stage during feeding with 3'-Me-DAB, we carried out radiorespirometry using $[1\text{-}^{14}\text{C}]$ glucose and $[6\text{-}^{14}\text{C}]$ glucose, and also determined the activity of G-6-PD. The use of these labelled compounds gave more clear-cut results than the previous radiorespirometric analysis using $[\text{U}\text{-}^{14}\text{C}]$ glucose. That is to say, three parameters of the radiorespirometric pattern were activated at the 4th week after the start of administration of 3'-Me-DAB. These changes coincided well with that of G-6-PD activity. Furthermore, high proliferative activity was found at this stage in terms of the incorporation of $[^3\text{H}]$ thymidine.

In a series of reports²¹⁻²³⁾ entitled "Precancerous Changes in Rat Liver during the Early Stage of 3'-Methyl-4-(dimethylamino) azobenzene Carcinogenesis", Onoe, et al. reported that pronounced degeneration of hepatocytes occurred, and the original hepatocytes gradually disappeared, being replaced by a proliferation of cholangiolar cells (the so called oval cells) after 2 weeks of ingestion of 0.06% 3'-Me-DAB. Oval cell proliferation reached a maximum at the 4th week after the administration of 3'-Me-DAB. Another interesting finding at this stage is a transient appearance of α -fetoprotein in the sera of rats, as reported by Watabe.¹⁷⁾ The elevation of glucose metabolism at this stage may be due to activation of the non-glycolytic pathway together with extensive cell proliferation. While, inhibitions of glucose metabolism by the 2nd week after feeding with 3'-Me-DAB may be mainly due to inactivation of glycolytic enzymes by the toxic effects of this carcinogen. Similar results²⁴⁻²⁵⁾ were obtained in cases of acute liver injury due to CCl_4 , and in regenerating rat liver.

It seems desirable to extend the present work to an investigation of glucose metabolism in the newly formed hepatocytes.

References

- 1) Kojima, S., Shiki, Y., and Kubodera, A.: *Radioisotopes*, **27**, (2) 78 (1979)
- 2) Glock, G.E., and McLean, P.: *Biochem. J.*, **55**, 400 (1953)
- 3) Glock, G.E., and McLean, P.: *ibid.*, **56**, 171 (1954)
- 4) Schmitz, H., Potter, V.R., and Hurbert,

- R.B.: *Cancer Res.*, **14**, 66 (1954)
- 5) Busch, H., Fujiwara, E., and Keer, L.M.: *ibid.*, **20**, 50 (1960)
 - 6) Bottomley, R.H., Pitot, H.C., and Potter, V.P.: *ibid.*, **23**, 400 (1963)
 - 7) Morris, H.P., and Weber, G.: *ibid.*, **23**, 987 (1963)
 - 8) Sharma, R.M., Sharma, C., Donnelly, A.J., Morris, H.P., and Weinhouse, S.: *ibid.*, **25**, 193 (1964)
 - 9) Shonk, C.E., Morris, H.P., and Boxer, G.E.: *ibid.*, **25**, 671 (1964)
 - 10) Gumaa, K.A., and McLean, P.: *Biochem. J.*, **115**, 1009 (1969)
 - 11) Hori, S.H., and Sasaki, M.: *Cancer Res.*, **29**, 880 (1969)
 - 12) Poirier, L.A., and Pitot, H.C.: *ibid.*, **30**, 1974 (1970)
 - 13) Watanabe, A., and Taketa, K.: *J. Biochem.*, **73**, 771 (1973)
 - 14) Taketa, K., Tanaka, A., and Watanabe, A.: *Proc. Symp. Chem. Physiol. Pathol.*, **11**, 30 (1971)
 - 15) Glock, G.E., and McLean, P.: *Biochem. J.*, **61**, 390 (1955)
 - 16) Field, J.B., Pastan, I., Johnson, P., and Herring, B.: *J. Biol. Chem.*, **235**, 1863 (1960)
 - 17) Watabe, H.: *Cancer Res.*, **31**, 1192 (1971)
 - 18) Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J.: *J. Biol. Chem.*, **193**, 256 (1951)
 - 19) Schmidt, G., and Thannhauser, S.J.: *ibid.*, **161**, 83 (1945)
 - 20) Schneider, T.W., Bushnell, D.E., and Potter, V.R.: *Cancer Res.*, **30**, 1867 (1970)
 - 21) Iwasaki, T., Dempo, K., Kaneko, A., and Onoe, T.: *Gann*, **63**, 21 (1972)
 - 22) Kaneko, A., Dempo, K., Iwasaki, T., and Onoe, T.: *ibid.*, **63**, 31 (1972)
 - 23) Kaneko, A., Dempo, K., and Onoe, T.: *ibid.*, **63**, 41 (1972)
 - 24) Kojima, S., and Kubodera, A.: *Jap. J. Nucl. Med.*, **16**, (2) 141 (1979)
 - 25) Kojima, S., and Kubodera, A.: *Radioisotopes*, in preparation

要 旨

3'-Methyl-4-(dimethylamino) azobenzene ラット投与期間中における グルコース代謝のラジオレスピロメトリーによる解析

——〔1-¹⁴C〕 glucose を用いたラジオレスピロメトリー——

小島周二, 石井長敏*, 志気保子*, 久保寺昭子*

帝京大学薬学部放射薬品化学

199-01 神奈川県津久井郡相模湖町寸沢嵐1091-1

*東京理科大学薬学部放射薬品化学

162 東京都新宿区市ヶ谷船河原町12

3'-Methyl-4-(dimethylamino) azobenzene (3'-Me-DAB) 投与初期において五炭糖リン酸回路による糖代謝の活性化をわれわれはさきの報告にて予測した。今回、前がん状態肝における、この非解糖系回路による糖代謝の活性化を、1 および 6 位標識グルコースを用いたラジオレスピロメトリーにて明らかにした。〔1-¹⁴C〕 glucose によるラジオレスピロメトリーにおいて、peak time は 3'-Me-DAB 投与後 2 週目まで著しく遅れたが、3 週目ではほぼ正常値を示し、逆に 4 週目で早まり、5, 6 週目ではふたたび正常の速さとなった。peak height, yield value はいずれも peak time の変動とともに変化し、早まるさいには増加し、遅れるさいには低下した。一方呼吸排泄 ¹⁴CO₂ の C-1/C-6 比は 4 週目で 1.52 まで上昇した。また G-6-PD 活性も 4 週目で最高値を示した。