Short note

An enzymatic assay for myo-inositol in tissue samples

Naoki Ashizawa, Motoyuki Yoshida*, Tomoji Aotsuka

Pharmaceutical Research Laboratory, Research Center, Research and Development Division, Grelan Pharmaceutical Co. Ltd., 3-4-3 Sakaecho, Hamura, Tokyo 205-0002, Japan

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Abstract

An enzymatic assay for myo-inositol (MI) was modified. The method is based on the oxidation of MI by NAD⁺-dependent MI dehydrogenase, coupled to reoxidation of NADH by iodonitrotetrazolium chloride and diaphorase. The resultant formazan is measured spectrophotometrically. In order to remove interference by glucose, preliminary phosphorylation of glucose by hexokinase was employed before the above reaction. The assay is quantitative for MI in amounts ranging from 1 to 20 nmol. This method gives a negligible blank, even in the measurement of rat serum. The reduced MI content in the sciatic nerve and lens of streptozotocin-induced diabetic rats recovered in a dose-dependent manner by treatment with a novel potent aldose reductase inhibitor, GP-1447 [3-{(4,5,7-trifluorobenzothiazol-2-yl)methyl}-5-methylphenylacetic acid]. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

myo-Inositol (MI) is indispensable in many physiological processes, and alteration of its metabolism is assumed to be involved in various diseases [1]. For the determination of MI in tissues, gas chromatography is most commonly used. MacGregor and Matschinsky have developed an enzymatic assay [2]. It is based on the oxidation of MI...
and the reduction of NAD$^+$ by MI dehydrogenase (MIDH) coupled with the scavenge reaction catalyzed by malate dehydrogenase to reoxidize the NADH produced in the first reaction. The resultant malate, which is stoichiometric with MI, is measured fluorometrically. Although the method is highly sensitive, it gives a high blank in the measurement of serum [2] and cells [3] due to endogenous malate. We present a modified method of MacGregor, i.e., the second reaction is the conversion of iodonitrotetrazolium chloride (INT) to formazan by diaphorase. Consequently, this method gives a low blank even in the measurement of rat serum.

Using the modified method, effects of aldose reductase inhibitors on MI content in tissues of streptozotocin (STZ)-induced diabetic rats were investigated.

2. Materials and methods

2.1. Measurement of myo-inositol (MI) content in tissues

The MI content in tissues was measured enzymatically following the oxidation of MI by NAD$^+$-dependent MIDH, coupled to reoxidation of NADH with INT and diaphorase. The resultant formazan was measured spectrophotometrically.

Serum (250 μl) was deproteinized with 250 μl (16%, w/v) perchloric acid and centrifuged at 5000×g for 10 min at 4°C. Sciatic nerve and lens were homogenized in 600 μl (16%, w/v) perchloric acid and centrifuged. All supernatants were neutralized with 2.0 M K₂CO₃ and centrifuged again. The resultant supernatant was forwarded to glucose phosphorylation.

A 100-μl aliquot of the supernatant was added to 10 μl hexokinase reagent (200 mM Tris–HCl buffer, 400 mM adenosine triphosphate disodium (ATP-2Na) (the pH of the solution was adjusted to 8.6 by adding 10.0 M NaOH) and 115 U/ml hexokinase). The mixture was incubated at 37°C for 90 min, and then heated for 3 min in a boiling water bath to stop the reaction. Endogenous NADH and NADPH were then eliminated by adding 20 μl of 4.5 M HCl. After 10 min at 25°C, 22 μl of 3.0 M K₂CO₃ was added for neutralization.

Measurements were done using a 96-well microplate. One hundred μl of tissue extract obtained as described above were mixed with 100 μl of MI reagent, which contained 210 mM triethanolamine hydrochloride–32 mM K₂HPO₄–KOH buffer (pH 8.6), 1.2% (v/v) Triton X-100, 10 mM β-NAD, 1.0 U/ml diaphorase, 0.1% (w/v) bovine serum albumin, 60 μg/ml INT. After the absorbance of the solution was measured at 492 nm with a microplate reader (model MPR-A4i, Tosoh, Tokyo, Japan), the reaction was
initiated by addition of 10 μl 2.1 U/ml MIDH dissolved in 20 mM potassium phosphate buffer (pH 7.0) to each well. The mixture was allowed to stand for 30 min at room temperature, then the absorbance (A) at 492 nm was measured again. From ΔA, an increase in absorbance during the reaction, MI content was calculated.

2.2. Induction of diabetes and treatment with drugs

Male Sprague-Dawley rats (5 weeks of age) were purchased from Japan SLC, and used for the study after an acclimatization period of 1 week. After an overnight fast, rats were subjected to a single intravenous injection of 60 mg/kg of STZ, immediately after dissolution in saline, to induce diabetes. Normal control rats were administered saline in a volume of 1 ml/kg instead of STZ. After the establishment of diabetes was confirmed by measuring non-fasting serum glucose, aldose reductase inhibitors were orally administered to diabetic rats once daily for 2 weeks. Twenty-four hours after the final dosing, rats were sacrificed under ether anesthesia. Sciatic nerve and lens were weighed immediately and frozen at −80°C until determination of MI content.

2.3. Drugs

GP-1447 [4,5] and SNK-860 [6] were synthesized at the Research and Development Division of Grelan Pharmaceutical. Both compounds were suspended in an aqueous solution of 0.5% (w/v) sodium carboxymethylcellulose. MI was purchased from Wako Pure Chemicals (Osaka, Japan). STZ, hexokinase, ATP-2Na, β-NAD, diaphorase, bovine serum albumin, INT, MIDH and glucose-6-phosphate were obtained from Sigma (St. Louis, MO, USA).

3. Results and discussion

3.1. Effects of conditions of the assay

The measurement of MI followed the protocol of MacGregor and Matschinsky [2] and Shayman et al. [3] with some modifications. Two parts were modified for higher spectrometric response. First, triethanolamine hydrochloride–KH₂PO₄–KOH buffer (pH 8.6) provided higher absorbance than the Tris–HCl buffer (pH 9.0) used previously [2,3]. Second, the increased concentration of β-NAD in the reaction mixture to 4.8 mM also allowed for higher absorbance.

Glucose at a concentration above 1 mM demonstrated significant reactivity as described previously [7]. In the previous report, disappearance of high concentration glucose was performed in two steps, i.e., enzymatic conversion and treatment with NaOH [2]. The reaction with hexokinase used in this study completely phosphorylated glucose to glucose-6-phosphate even at 30 mM, which is equivalent to the concentration in serum from diabetic animals, leading to the removal of interference of glucose with the assay. The ΔA values obtained by the reaction with and without 30 mM glucose in sciatic nerve extract were 0.237 and 0.238, respectively. Furthermore, addition of 30
mM glucose-6-phosphate to the same extract after hexokinase treatment provided ΔA of 0.248, indicating no significant reactivity.

The MI standard curve used for the determination in the sciatic nerve and lens was linear in amounts ranging from 1 to 20 nmol (Fig. 1). It was obtained by addition of MI to the neutralized perchloric acid solution followed by hexokinase treatment. MI in amounts ranging from 1 to 20 nmol was linear. The standard curve was reproduced in the presence of 30 mM glucose (data not shown).

Tissue blanks contained tissue samples, to which were added MI reagent lacking MIDH. Although absorbance of the blanks was slightly increased during the reaction, the increase in absorbance of the blanks was not affected by the presence of extract from any tissue. This indicates that the blanks for tissues, including serum, are negligible. This is in marked contrast to the results obtained by the previous method, in which the blanks of serum and cells reached 45 and 20–30% of the measured MI content, respectively [2,3]. The concentrations of MI in serum from normal rats and STZ-induced diabetic rats were 69.7 ± 5.2 μM (n = 7) and 62.9 ± 4.6 μM (n = 7), respectively.

3.2. Effects of treatment with aldose reductase inhibitors on MI content in tissues of diabetic rats

MI level in tissue extract measured in the present study agreed well with literature values [2]. MI content in the sciatic nerve of STZ-induced diabetic rats was decreased by
Table 1
myo-Inositol content in the sciatic nerve (Exp. 1) and lens (Exp. 2) of normal and streptozotocin-induced diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg per day)</th>
<th>n</th>
<th>Sciatic nerve</th>
<th>Lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>–</td>
<td>7</td>
<td>3.43±0.27**</td>
<td></td>
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<tr>
<td>Diabetic control</td>
<td>–</td>
<td>6</td>
<td>1.94±0.24</td>
<td></td>
</tr>
<tr>
<td>SNK-860</td>
<td>0.3</td>
<td>6</td>
<td>1.34±0.29</td>
<td></td>
</tr>
<tr>
<td>SNK-860</td>
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<td>6</td>
<td>2.35±0.17</td>
<td></td>
</tr>
<tr>
<td>SNK-860</td>
<td>3</td>
<td>6</td>
<td>2.00±0.31</td>
<td></td>
</tr>
<tr>
<td>GP-1447</td>
<td>0.3</td>
<td>6</td>
<td>2.52±0.49</td>
<td></td>
</tr>
<tr>
<td>GP-1447</td>
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<td>6</td>
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<td></td>
</tr>
<tr>
<td>GP-1447</td>
<td>3</td>
<td>5</td>
<td>3.50±0.32**</td>
<td></td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>–</td>
<td>7</td>
<td>1.79±0.11**</td>
<td></td>
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<tr>
<td>Diabetic control</td>
<td>–</td>
<td>8</td>
<td>0.11±0.02</td>
<td></td>
</tr>
<tr>
<td>SNK-860</td>
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<td>7</td>
<td>0.22±0.06</td>
<td></td>
</tr>
<tr>
<td>SNK-860</td>
<td>10</td>
<td>8</td>
<td>0.67±0.06*</td>
<td></td>
</tr>
<tr>
<td>GP-1447</td>
<td>3</td>
<td>7</td>
<td>0.61±0.09*</td>
<td></td>
</tr>
<tr>
<td>GP-1447</td>
<td>10</td>
<td>7</td>
<td>1.05±0.10**</td>
<td></td>
</tr>
</tbody>
</table>

* n refers to number of rats.

In experiment 1, following a 4-week period of untreated diabetes, each compound was orally administered to male Sprague-Dawley rats once daily for 2 weeks. In experiment 2, following a 1-week period of untreated diabetes, each compound was orally administered to male Sprague-Dawley rats once daily for 2 weeks. Values are the means±S.E.

Significantly different from diabetic control group: *P<0.05; **P<0.01, using Dunnett’s test.

43% as compared with normal rats (Table 1, Exp. 1). Treatment for 2 weeks with a novel aldose reductase inhibitor, GP-1447, dose-dependently reversed the reduced MI content with an ED50 value of 0.47 mg/kg per day. On the other hand, SNK-860 did not induce significant restoration of MI in contrast to the previous report [6]. The discrepancy between the studies may be due to the difference in dosing protocols. In the study of Mizuno et al. [6], administration of SNK-860 was started 2 weeks after the induction of diabetes, while in the present study, treatment with the compound for the same duration was initiated 4 weeks after the STZ-injection. The longer period of untreated diabetes is considered to cause greater deterioration in sugar metabolism, leading to a poor recovery of MI content.

The MI in the lens was almost completely depleted by the induction of diabetes (Table 1, Exp. 2). Treatment with SNK-860 or GP-1447 partially restored the decrease in MI content in the lens in a dose-dependent manner. GP-1447 was estimated to be three times more potent than SNK-860 in increasing MI content in the lens.

Although the assay is less sensitive than the method of MacGregor and Matschinsky [2], it is adequately sensitive for measuring MI in serum or tissue weighing 1 mg or more. The method is not tedious since no special preparation of tissue sample is required and interference of endogenous glucose is completely removed in one step. Furthermore,
because of the negligible blank, net MI content even in the serum can be measured without employing blanks.

References