Inhibition of glycogenolysis in primary rat hepatocytes by 1,4-dideoxy-1,4-imino-D-arabinitol

Birgitte ANDERSEN¹, Andreas RASSOV, Niels WESTERGAARD and Karsten LUNDGREN²

Department of Diabetes Biochemistry and Metabolism, Novo Nordisk A/S, Building C9.S19, Novo Nordisk Park, DK-2760 Måløv, Denmark

1,4-Dideoxy-1,4-imino-D-arabinitol (DAB) was identified previously as a potent inhibitor of both the phosphorylated and non-phosphorylated forms of glycogen phosphorylase (EC 2.4.1.1). In the present study, the effects of DAB were investigated in primary cultured rat hepatocytes. The transport of DAB into hepatocytes was dependent on time and DAB concentration. The rate of DAB transport was 192 pmol/min per mg of protein per mM DAB_{medium-concentration}. In hepatocytes, DAB inhibited basal and glucagon-stimulated glycogenolysis with IC₅₀ values of 1.0 ± 0.3 and 1.1 ± 0.2 μM, respectively. The primary inhibitory effect of DAB on glycogenolysis was shown to be due to inhibition of glycogen phosphorylase but, at higher concentrations of

DAB, inhibition of the debranching enzyme (4-α-glucanotransferase, EC 2.4.1.25) may have an effect. No effects on glycogen synthesis were observed, demonstrating that glycogen recycling does not occur in cultured hepatocytes under the conditions tested. Furthermore, DAB had no effects on phosphorylase kinase, the enzyme responsible for phosphorylation and thereby activation of glycogen phosphorylase, or on protein phosphatase 1, the enzyme responsible for inactivation of glycogen phosphorylase through dephosphorylation.

Key words: glycogen phosphorylase, glycogen synthesis, phosphorylase kinase, PP1.

INTRODUCTION

In non-insulin-dependent diabetes mellitus (type-II diabetes) hepatic glucose production is increased [1]. Hepatic glucose is produced from two pathways: glycogenolysis (breakdown of glycogen) and gluconeogenesis (*de novo* synthesis of glucose). A number of studies have measured the relative contributions of these pathways, but they have been difficult to ascertain and studies have resulted in varying estimates [2–7]. Nevertheless, glycogenolysis may account for more than 70% of the hepatic glucose production both in healthy controls [6] and in patients with type-II diabetes [7]. Furthermore, recent studies suggest that a substantial portion of gluconeogenesis appears to be cycled through the glycogen pool prior to efflux from the liver cells [8]. Therefore, a possible way to suppress hepatic glucose production and lower blood glucose in type-II diabetes patients may be through inhibition of hepatic glycogen phosphorylase (GP, EC 2.4.1.1) [9].

Hepatic glycogen metabolism is regulated through phosphorylation and dephosphorylation of the two rate-limiting enzymes, GP and glycogen synthase (GS, EC 2.4.1.11). Phosphorylation and activation of GP are stimulated by glucagon and adrenaline and are mediated via protein kinase A [10]. Dephosphorylation of GP is mediated by protein phosphatases, including protein phosphatase 1 (PP1) [11,12]. The phosphorylated form of GP is termed GP_a and the non-phosphorylated form GP_b. Furthermore, allosteric regulators of GP are well described. Glucose, which binds to the active site, inactivates GP_a by competitive inhibition with glycogen and by stabilizing the less active T-state, which is also a better substrate for PP1 [13,14]. In addition, caffeine, a model ligand, inhibits GP_a by binding to the nucleoside-inhibitor site [15]. In contrast, AMP activates GP_b by binding to the nucleotide-activator site [13]; the liver isoform of GP_b can be activated to around 20%

of the GP_a activity, whereas the muscle isoform can be activated to 80% of the GP_a activity.

Dephosphorylation of GS is stimulated by insulin and is mediated by type-1 protein phosphatase bound to glycogen (PP-1G) [16]. This process is secondary to dephosphorylation of GP_a, which relieves the allosteric inhibitory effect of GP_a on PP-1G [17,18]. It has been suggested that this well-defined antagonistic regulatory mechanism fails under conditions in which the amount of glycogen is limited [19]. Thus under these conditions GP_a is unable to inhibit PP-1G, which would allow a dephosphorylation of GS, resulting in concurrent activity of GP_a and phosphorylated GS (GS_a) and futile cycling.

Several pharmacological GP inhibitors have been described in the literature. Some glucose analogues substituted in the C-1 position have been shown to be more potent inhibitors (4–50-fold) of muscle GP compared with glucose, but are less potent inhibitors of liver GP [9,20–22]. Another potent muscle GP_b inhibitor (Bay W1807), binding to the AMP site of muscle GP, has been identified [23]. But again it is only a weak inhibitor of GP_a, the active form in the liver. More recently, however, a potent inhibitor (CP-91149) of liver GP was described. This compound was shown to bind to the nucleoside-inhibitory site of GP *in vitro* and, moreover, to normalize blood glucose after chronic administration to spontaneously diabetic *ob/ob* mice [24].

In enzyme assays, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) was shown to be a potent inhibitor of liver GP (K. Fosgerau, M. Kristiansen, N. Westergaard, P. Justesen, E. G. Mortensen and K. Lundgren, unpublished work). Here the effect of DAB is investigated in primary rat hepatocytes, and DAB is shown to be the most potent inhibitor of basal and glucagon-stimulated glycogenolysis ever reported.

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Abbreviations used: DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; GP, glycogen phosphorylase; GP_a, GP_b, phosphorylated and non-phosphorylated forms of GP respectively; GS, glycogen synthase; PP1, protein phosphatase 1; PP-1G, type-1 protein phosphatase bound to glycogen.

¹ To whom correspondence should be addressed (e-mail BttA@Novo.dk).

² Present address: Insmed Pharmaceuticals Inc., 800 E. Leigh St., Richmond, VA 23219, U.S.A.

MATERIALS AND METHODS

Materials

Male Wistar rats were obtained from Møllegaard Breeding Center (Lille Skensved, Denmark). Tissue culture flasks and dishes were purchased from Nunc A/S (Roskilde, Denmark). Collagenase type IV, collagen type I (rat tail), dexamethasone, glucose oxidase kit, rabbit muscle GPb and all other enzymes and chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Medium 199, foetal calf serum, Trypan Blue, penicillin and streptomycin were purchased from Life Technologies (Roskilde, Denmark). [^3H]-DAB (sp. radioactivity 4 Ci/mmol) was synthesized as published previously [25]. Human insulin and glucagon were from Novo Nordisk A/S. Scintillation fluid Ultima Gold was from Packard Instrument (Meriden, CT, U.S.A.). The protein-assay kit was from Bio-Rad (Copenhagen, Denmark). Partially purified rabbit skeletal-muscle PP1 was purchased from Upstate Biotechnology (TriChem Aps, Virum, Denmark). [γ - ^{33}P]ATP (sp. radioactivity 2500 Ci/mmol) and ^{14}C -methylated molecular-marker proteins and other radiolabelled chemicals were from Amersham Life Science (Little Chalfont, Bucks, U.K.). Tris/glycine gels (8%), SDS loading buffer and NuPage Mes SDS running buffer were from Novex, purchased at Kenentec (Copenhagen, Denmark).

Isolation and culturing of hepatocytes

Male Wistar rats (≈ 200 g) were anaesthetized with a freshly prepared mixture (1:1) of Hypnorm (0.05 mg/ml fentanyl/2.5 mg/ml flubazone) and Dormicum (1.25 mg/ml midazolam) administered subcutaneously (1 ml/kg). Hepatocytes were isolated from rats fed *ad libitum* by a two-step perfusion technique essentially as described by Seglen [26]. Cell viability, assessed by Trypan Blue exclusion, was consistently greater than 80%. Cells were plated on to collagen-coated six-well plates in basal medium (Medium 199, 5.5 mM glucose supplemented with 0.1 μM dexamethasone, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 1 nM insulin) with 4% foetal calf serum at a cell density of 10^6 cells/well. The medium was replaced with basal medium 1 h after initial plating in order to remove dead cells. For transport experiments and intracellular-volume determinations the medium was changed after 24 h to basal medium, and then transport experiments were performed 24 h later. For glycogenolysis experiments and intracellular-volume determinations medium was changed after 24 h to basal medium supplemented with 9.5 mM glucose and 19 nM insulin, and experiments were then performed 20 h later. For glycogen-synthesis experiments medium was changed after 24 h to basal medium supplemented with different concentrations of glucose and insulin, as described in the text, and experiments were terminated 24 h later.

Protein contents in the culture dishes were determined after solubilization in 0.1 M NaOH using the Bio-Rad protein-assay kit and human serum albumin as standard.

Glycogenolysis

Hepatocytes (10^6 cells, equalling ≈ 1 mg of protein) were washed twice with prewarmed (37 °C) buffer A (117.6 mM NaCl, 5.4 mM KCl, 0.82 mM MgSO_4 , 1.5 mM KH_2PO_4 , 20 mM Hepes, 9 mM NaHCO_3 , 0.1% w/v human serum albumin and 2.25 mM CaCl_2 , pH 7.4 at 37 °C) and incubated in 1 ml of buffer A containing increasing concentrations of DAB with or without 0.5 nM glucagon for 70 min. Basal and glucagon-induced glycogenolysis were measured as glucose released into buffer A, using a glucose oxidase kit. Glycogen levels were determined after two washes of cells with ice-cold 0.9% (w/v) NaCl by amyloglucosidase (exo-

1,4- α -D-glucosidase, EC 3.2.1.3) digestion, as described by Gomez-Lechon et al. [27]. Lactate in the medium was determined using Sigma lactate reagent (735-10).

The effect of 1-deoxynojirimycin on glucagon-stimulated glycogenolysis was measured as glucose released into buffer A over 90 min, as described above.

Glycogen synthesis

Glycogen synthesis was determined as an increase in glycogen levels. Cells (10^6 cells, ≈ 1 mg of protein) were incubated for 24 h in 1 ml of basal medium with increasing concentrations of glucose (5.5 and 15 mM) and insulin (1 and 10 nM), and with or without 10 μM DAB, which was added twice, at 0 and 12 h. After two washes with cold 0.9% (w/v) NaCl, glycogen was digested by amyloglucosidase as described above.

Phosphatase activity

Purified rabbit muscle GPb was phosphorylated by glycogen phosphorylase kinase as described by Antoniow et al. [28]. The phosphorylated GPa was separated from remaining [γ - ^{33}P]ATP by Ultrafree-MC filters (10000 NMWL Filter Unit) in an Eppendorf centrifuge. The rotor was operated for 60 min at 4 °C and 2000 g. Radiolabelled substrate [^{33}P]GPa was dephosphorylated according to Nyomba et al. [29]. Dephosphorylation was carried out by incubating the radiolabelled GPa with 0.1 unit of rabbit skeletal-muscle PP1 for 5 min at 30 °C in a buffer containing 50 mM Tris, 1.0 mM EDTA, 12.5 mM β -mercaptoethanol and 0.5 mg/ml BSA (pH 7.0–7.5) in a final volume of 70 μl . The reaction was terminated by adding 100 μl of 17.5% (v/v) trichloroacetic acid and 100 μl of 6 mg/ml BSA and placing on ice. Denatured proteins were removed by centrifugation for 4 min at 4 °C and 10000 g. The amount of radioactivity was determined in 200 μl of the supernatant. All samples were corrected for [^{33}P]P_i radioactivity measured in the supernatant in reactions carried out in the absence of PP1. This was considered to be non-specific and not due to specific dephosphorylation of [^{33}P]GPa.

Phosphorylase kinase activity

Purified rabbit muscle GPb (6 units) was phosphorylated by skeletal-muscle glycogen phosphorylase kinase (1.5 units) in a buffer containing 50 mM Tris (pH 8.0), 50 mM glycerol 2-phosphate, 10 mM magnesium acetate and 1 mM ATP traced with [γ - ^{33}P]ATP (0.05 $\mu\text{Ci}/\mu\text{l}$) and varying concentrations of DAB or 50 nM staurosporine for 30 min at 25 °C in a final volume of 50 μl . Reactions were terminated by adding SDS loading buffer and placing on ice. The reaction (25 μl) was loaded on to an Tris/glycine 8% gel and run for 40 min at constant voltage (200 V) in a NuPage Mes SDS buffer. ^{14}C -Methylated proteins were loaded as molecular markers. The gel was transferred to a PhosphorImager screen cassette and after ≈ 30 min of exposure the bands were quantified on a PhosphorImager from Molecular Dynamics.

Transport assay

All transport studies were initiated by washing hepatocytes (10^6 cells, ≈ 1 mg of protein) twice with prewarmed (37 °C) buffer A. Time studies were performed by incubating hepatocytes in 1 ml of buffer A containing 0.1, 1 or 10 mM DAB traced with [^3H]DAB (0.5 $\mu\text{Ci}/\text{ml}$) at 37 °C for various times. Concentration-dependent transport studies were performed by incubating hepatocytes for 3 min at 37 °C in 1 ml of buffer A containing

Figure 1 Effect of DAB on basal and glucagon-stimulated glycogenolysis

Hepatocytes were incubated in the presence of 15 mM glucose and 10 nM insulin for 20 h.

increasing concentrations of DAB traced with [3 H]DAB (0.5 μ Ci/ml). For competition experiments, hepatocytes were incubated in 1 ml of buffer A containing [3 H]DAB (0.5 μ Ci/ml) and 10 mM substrates. Temperature-dependent transport studies were performed by incubating hepatocytes in buffer A containing [3 H]DAB (0.5 μ Ci/ml) at either 4 or 37 °C for 3 min. The effect of 2,4-dinitrophenol was determined by pre-incubating hepatocytes with 100 μ M 2,4-dinitrophenol for 10 min and then transport experiments were initiated in buffer A containing [3 H]DAB (0.5 μ Ci/ml) at 37 °C for 3 min. Uptake of DAB was terminated by three quick washes with ice-cold 0.9 % (w/v) NaCl. Cells were lysed with 1 M NaOH, scintillation fluid was added and [3 H]DAB was measured. All experiments were corrected for radioactivity present in culture briefly exposed (1–2 s) to [3 H]DAB (0.5 μ Ci/ml).

Intracellular volume

The intracellular volume of the hepatocytes was determined by washing hepatocytes (10^6 cells, $\approx$ 1 mg of protein) twice in prewarmed buffer A. The hepatocytes were incubated in 1 ml of buffer A containing 1 μ Ci/ml [14 C]3-*O*-methylglucose for 10 min (the [14 C]3-*O*-methylglucose uptake was in equilibrium) and subsequently washed twice in ice-cold buffer A containing 1 mM phloretin to avoid efflux. The cells were lysed in 1 M NaOH, scintillation fluid was added and [14 C]3-*O*-methylglucose was measured.

Statistical analyses

Statistical analyses were performed using a two-tailed two-sample Student's *t* test (homoscedastic or heteroscedastic, depending upon variance) performed in Microsoft Excel 7.0. Statistical significance was determined to be $P < 0.05$. Replicates (*n*) were defined as separate hepatocyte preparations and in phosphorylase kinase assays and in PP1 assays as separate determinations.

RESULTS AND DISCUSSION

Since DAB has previously been shown to be a potent inhibitor of extracted liver GPs (K. Fosgerau, M. Kristiansen, N. Westergaard, P. Justesen, E. G. Mortensen and K. Lundgren, unpublished work), the present study was undertaken to characterize the effect of this compound in primary cultures of rat hepatocytes. The data revealed that DAB was a highly potent inhibitor of both basal and glucagon-stimulated glucose production (Figure 1a). As seen in Figure 1(a), 0.5 nM glucagon increased the glucose released into the medium by $\approx$ 2.3 fold. From the release of glucose into the medium, the IC_{50} values for inhibition of basal and glucagon-stimulated glucose production were found to be 1.0 ± 0.3 and 1.1 ± 0.2 μ M, respectively. During glycogenolysis a significant amount of lactate is produced. In the presence of increasing concentrations of DAB a reduction in lactate production was observed (Figure 1b). The IC_{50} values for this inhibition correlate with the IC_{50} values obtained from the inhibition of the glucose production. The lower lactate levels in

(a) Subsequently, basal (■) and glucagon-induced (0.5 nM glucagon; ●) glucose production were measured over 70 min (*n* = 5). (b) Basal (■) and glucagon-induced (0.5 nM; ●) lactate production were measured over 70 min (*n* = 3). (c) After glycogenolysis, glycogen levels after basal (■) and glucagon-induced (0.5 nM; ●) glycogenolysis were determined (nmol of glycogen/mg of protein; *n* = 3). The solid bar indicates the glycogen level before start of the experiment (20 h). All results are means $\pm$ S.E.M.

Table 1 Effect of DAB on basal and glucagon-induced glycogenolysis

The values in this Table are from Figure 1. The total C₆ carbon contents recovered were calculated as the sum of the glucose production and half the lactate production (C₃ carbon) and the glycogen found in the cells after glycogenolysis. To calculate how much of the initial glycogen could be recovered, the total C₆ carbon content was divided by the value of the initial glycogen content (2449 nmol/mg of protein).

DAB (μ M)	Glucagon (nmol)	Glucose (nmol/mg of protein)	Lactate (nmol/mg of protein)	Glycogen (nmol/mg of protein)	Total C ₆ (nmol/mg of protein)	Recovered (%)
0.0	0	274	598	1381	1954	80
0.2	0	236	525	1559	2058	84
0.5	0	211	490	1464	1920	78
2.0	0	115	348	1883	2172	89
5.0	0	79	203	2037	2217	91
20.0	0	49	75	2182	2269	93
0.0	0.5	641	380	1108	1938	79
0.2	0.5	538	321	1230	1928	79
0.5	0.5	463	290	1332	1940	79
2.0	0.5	260	141	1735	2066	84
5.0	0.5	172	67	2025	2230	91
20.0	0.5	81	28	2238	2333	95

Table 2 Effect of DAB on glycogen synthesis

Hepatocytes were incubated for 24 h in basal media. Following this, cellular glycogen content was measured after a further 24 h of incubation at the indicated glucose and insulin concentrations. Where present (+), DAB was added after 0 and 12 h. The glycogen level at the start of the incubation was determined as 150 ± 20 nmol of glycogen/mg of protein. Results are means $\pm$ S.E.M. ($n = 3$).

DAB (10 μ M)	Glucose (mM)	Insulin (nM)	Glycogen synthesis (nmol of glycogen/mg of protein)
—	5.5	1	190 ± 55
+	5.5	1	224 ± 65
—	15.0	1	1458 ± 390
+	15.0	1	1551 ± 120
—	5.5	10	498 ± 145
+	5.5	10	481 ± 133
—	15.0	10	3343 ± 170
+	15.0	10	3435 ± 164

the presence of glucagon are in agreement with glucagon's inhibitory effect on glycolysis (e.g. pyruvate kinase) [30]. Furthermore, the measurements of cellular glycogen content confirmed that glycogenolysis was inhibited by DAB (Figure 1c). The IC₅₀ values related to this inhibition correlate with the values mentioned above.

Lactate levels were approximately a factor of two higher in the absence of glucagon, explaining the almost similar rates of glycogenolysis independent of glucagon (Figure 1c). The high glycolytic rate in the absence of glucagon can be explained by the lack of glucose in the incubation medium. More than 90 % of the initial glycogen level was recovered in the presence of 20 μ M DAB (Table 1), whereas only ≈ 80 % was recovered in the absence of or at lower concentrations of DAB. This is in agreement with a higher rate of glycolysis and thereby of CO₂ production.

Since DAB is an inhibitor of GP, and since GS is activated secondarily to inactivation of GP, we tested the effect of DAB on glycogen synthesis. Both insulin and glucose showed effects on glycogen synthesis; however, at different insulin and glucose concentrations glycogen stores were unaffected by DAB (Table 2), demonstrating that glycogen synthesis was not directly affected by DAB. Since glycogen levels were not significantly greater in the presence of DAB, these experiments also demon-

strated that there was no simultaneous formation and degradation of glycogen (futile cycling) under the conditions tested. The findings are in disagreement with data reported by Stalmans et al. [31]. They, however, used freshly isolated hepatocytes from fasted rats. Grunnet et al. [32], who, like us, used cultured hepatocytes, also failed to demonstrate glycogen recycling.

Previously, DAB was also shown to be a weak inhibitor of mammalian glucosidases (IC₅₀ for rat liver glucosidase, ≈ 20 μ M) [33,34]. Therefore, it was possible that inhibition of glycogenolysis was mediated through inhibition of the debranching enzyme, which is an α -1,6-glucosidase (4- α -glucanotransferase). This hypothesis was tested in a glycogenolysis assay using the potent α -glucosidase inhibitor 1-deoxynojirimycin (IC₅₀ determined as 1 μ M), which enters the cell easily [35]. The glucagon-stimulated glycogenolytic rate obtained in the presence of 50 μ M 1-deoxynojirimycin was 59.3 ± 7.1 % ($n = 3$) compared with 100 % in the absence of 1-deoxynojirimycin. These data are consistent with those obtained by Bollen et al. [36]. Our data therefore suggest that DAB has an additional effect on the debranching enzyme at higher concentrations of DAB. However, since DAB inhibits glycogenolysis with an IC₅₀ value of 1 μ M, its primary effect must be on GP, since DAB inhibits rat liver α -glucosidase II with an IC₅₀ of 20 μ M.

Evidently, some inhibitors of GP α that are structurally related to glucose additionally inhibit glycogenolysis through an activation of PP1 [21]. It was therefore investigated whether DAB had any effect on muscle PP1. By using purified PP1 instead of liver extracts, binding and/or regulation of PP1 to or by glycogen-targeting proteins [37,38] was avoided. Using 0.1 unit of PP1 the dephosphorylation reaction was linear for the first 8 min (results not shown); therefore, following experiments were carried out over 5 min. The effects of glucose and DAB on PP1 were tested in the concentrations effective in primary hepatocytes. In contrast with glucose, which at 20 mM significantly ($P < 0.001$) increased the activity of PP1 by 1.8-fold, DAB had no effect on the activity of skeletal-muscle PP1 or the stabilization of the T-state of GP α . Likewise, 20 μ M DAB did not alter the glucose-induced activation of PP1. Due to its commercial availability, skeletal-muscle PP1 was used in these experiments, and the effect of DAB on liver PP1 is therefore unknown. However, glucose has previously been shown to activate both isoenzymes [21], suggesting that skeletal-muscle PP1 may be used as a valid substitute for the liver enzyme. Therefore, under these assumptions it appears unlikely that activation of PP1 by DAB would contribute to the inhibition of hepatic glycogenolysis.

Figure 2 Concentration-dependent uptake of DAB into hepatocytes

Hepatocytes were incubated for 3 min with increasing concentrations of DAB traced with $0.5 \mu\text{Ci/ml}$ [^3H]DAB. Results are means $\pm$ S.E.M. ($n = 3$).

Additional inhibition of phosphorylase kinase by DAB beyond inhibition of GP could inhibit glucagon-induced glycogenolysis. This possible influence of DAB on phosphorylase kinase was investigated. Skeletal-muscle phosphorylase kinase was used because it was commercially available. The phosphorylated protein bands were detected at a position equalling the 96-kDa molecular marker. The phosphorylation of GPb to GPa was linear for 60 min using 1.5 units of phosphorylase kinase, and the phosphorylation of GPb was furthermore linear between 0.3 and 3 units of phosphorylase kinase for 30 min (results not shown). The phosphorylation of GPb was inhibited by 60% by 50 nM staurosporine. At 20 μM DAB no effect was observed on phosphorylase kinase activity. Furthermore, possible conformational changes of GP caused by DAB did not inhibit the phosphorylation.

In order to investigate the transport mechanism, cellular uptake of DAB was determined in primary hepatocytes. The time course of DAB uptake was tested at three concentrations (0.1, 1 and 10 mM) of DAB and was found to be linear for the first 5 min (0.1 mM DAB, $R^2 = 0.9831$; 1 mM DAB, $R^2 = 0.971$; 10 mM DAB, $R^2 = 0.975$). After 15 min of incubation the uptake was in equilibrium in all three experiments and the intracellular concentration of DAB was calculated to equal the extracellular concentration of DAB at this time point. Concentration and competition experiments were therefore carried out over 3 min. The transport of DAB was linear ($R^2 = 0.993$) up to the maximal tested concentration of 40 mM (Figure 2), and the experiment therefore failed to demonstrate a saturable transport mechanism. By linear-regression analysis the rate of uptake was determined to be 192 pmol/min per mg of protein per mM DAB_{medium-concentration}. The intracellular volume of the primary hepatocytes incubated in 5.5 mM glucose and 1 nM insulin was determined to be ≈ 1.0 pl/cell. In cells incubated in 15.5 mM glucose and 10 nM insulin the volume was determined as 1.2 pl/cell in agreement with cellular water retention. At higher concentrations of DAB, e.g. 40 mM DAB, hyperosmolarity induced a 10% reduction in the intracellular volume of the hepatocytes and saturation of uptake was therefore not

Table 3 Transport of DAB into hepatocytes

Hepatocytes were incubated for 3 min at 37 or 4 $^{\circ}\text{C}$ with the indicated concentration of substrates traced with [^3H]DAB (0.5 $\mu\text{Ci/ml}$). Transport of 0.5 $\mu\text{Ci/ml}$ [^3H]DAB, equalling 0.1 μM DAB, was used as a control and was set to 100%. In statistical analyses the effects of the compounds were analysed versus the control. Statistical significance was evaluated and is denoted as follows: * $P < 0.05$ and *** $P < 0.001$. Results are means $\pm$ S.E.M. ($n = 4$).

Substrate	Transport (%)
2,4-Dinitrophenol (100 μM)	97 $\pm$ 13
DAB (10 mM)	18 $\pm$ 3***
α -D-Glucose (10 mM)	94 $\pm$ 10
α -D-Fructose (10 mM)	123 $\pm$ 16*
Isosfagmine (10 mM)	31 $\pm$ 6***
DAB (4 $^{\circ}\text{C}$; 0.1 μM)	37 $\pm$ 4***

attempted. Assuming that DAB is distributed within the same phase as 3-*O*-methyl glucose, a minimum intracellular concentration of DAB of 3 μM after treatment of hepatocytes with 5 μM DAB for 3 min can be estimated. This value is close to reported K_i values of DAB (0.3–0.4 μM) for hepatic GP (K. Fosgerau, M. Kristiansen, N. Westergaard, P. Justesen, E. G. Mortensen and K. Lundgren, unpublished work).

Whereas the lack of saturation kinetics of DAB transport into hepatocytes suggested that the uptake mechanism is mediated through passive diffusion, the highly hydrophilic nature of DAB pointed to the existence of a specific transporter. Therefore, an additional series of experiments was carried out in hepatocytes. The results of these are summarized in Table 3. First, it can be noticed that DAB transport was unaffected by the mitochondrial uncoupler 2,4-dinitrophenol. Second, competition experiments with compounds for which hepatic transporters are known did not result in decreased uptake of DAB. However, isosfagmine, a derivative of DAB, inhibited the uptake of DAB by 70% at 10 mM. Third, the transport of DAB was dependent on temperature. Therefore, these experiments failed to elucidate the nature of the putative transporter, but indicated that DAB is transported by a low-affinity transporter system.

In conclusion, we have demonstrated that DAB is transported into rat hepatocytes. In cultured hepatocytes, DAB was unable to affect glycogen synthesis over 24 h. In contrast, DAB potently inhibited hepatic glucose production from glycogenolysis through a direct inhibition of glycogen phosphorylase, since DAB had no effect on PP1 or the kinase involved in covalent modification of GP. The indole compound described by Martin et al. [24] was shown to inhibit liver cell glycogenolysis with an IC_{50} of 2.1 μM . With an IC_{50} value of 1 μM DAB is the most potent inhibitor of liver cell glycogenolysis ever reported.

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