D-*chiro*-Inositol Attenuates Epinephrine-stimulated Hepatic Glucose Output in the Isolated Perfused Liver Independently of Insulin

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Abstract

D-*chiro*-Inositol (DCI) is a cyclic sugar alcohol that evokes both antidiabetic and insulin sensitizing effects. Pharmacological administration of DCI has been shown to lower blood glucose in rat models of diabetes mellitus and enhance insulin sensitivity in humans with polycystic ovary syndrome (PCOS). We hypothesised that the antidiabetic effects of DCI could be due to inhibition of hepatic glucose output (HGO). To test this hypothesis, we perfused isolated rat livers either with buffer, *myo*-inositol, DCI, or insulin, and investigated their respective effects on the stimulation of HGO by epinephrine. We found that perfusion with 200 µM DCI attenuated epinephrine-stimulated HGO by 35 % over 30 min as compared to the buffer control perfusion ($p = 0.05$). By comparison, perfusion with 1 nM insulin attenuated epinephrine-stimulated HGO by 57 % ($p < 0.0001$). The glucose-lowering effects by DCI occurred independently of insulin and were specific to the DCI stereoisomer as 200 µM *myo*-inositol had no effect. These findings suggest that DCI could evoke its antidiabetic effects in vivo by inhibition of HGO. Further identification of the protein targets involved could open up new avenues to regulate hyperglycaemia with wider implications for the treatment of hepatic insulin resistance in PCOS.

Introduction

There is significant interest in glucose-lowering therapeutic strategies for diabetes mellitus, a disease, which if left untreated can lead to serious complications. One agent that evokes antidiabetic effects is the naturally-occurring cyclic sugar alcohol, D-*chiro*-inositol (DCI) [1], which attenuates hyperglycaemia in animal models of diabetes mellitus [2]. DCI administration also improves insulin sensitivity, hyperinsulinemia, and ovulatory function in patients with polycystic ovary syndrome (PCOS), a condition where insulin resistance is a primary feature [3]. By contrast, the stereoisomer, *myo*-inositol, which is ubiquitously found in all mammalian tissues and involved in a wide range of physiological functions, does not display these properties [1].

Body stores of DCI reportedly decrease in diabetes and obesity [4, 5], which may be in part due to the phenomenon of increased DCI renal excretion through currently undefined mechanisms [6]. Interestingly, abnormally increased DCI urinary excretion is also associated with PCOS suggesting that this condition is associated with diminished tissue availability of DCI [7]. Thus, impaired DCI action may be common to both diabetes mellitus and PCOS [1, 3].

Despite compelling evidence supporting the glucose-lowering effects of DCI supplementation in vivo, the main metabolic sites of action of DCI are unknown. Increased glucose disposal in skeletal muscle has been proposed as one mechanism whereby blood-borne DCI-mediators act on downstream intracellular metabolic targets [1]. One such mediator was isolated from liver and identified as pinitol, β-1,4 galactosamine (INS-2) [1]. In addition, DCI has been shown to act as an antioxidant by reducing reactive oxidative species in endothelial cells treated with high glucose [8] and protect against diabetic neuropathy in streptozotocin-induced diabetic mice [9].

We hypothesised that the glucose-lowering effects of DCI in diabetes mellitus could also be due to direct effects on the liver. Increased HGO is a significant contributor to elevated fasting glucose levels in diabetes mellitus and to the development of insulin resistance [10, 11]. Inhibition of HGO could therefore potentially account for DCI's antidiabetic effects as well as its sensi-

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Bibliography
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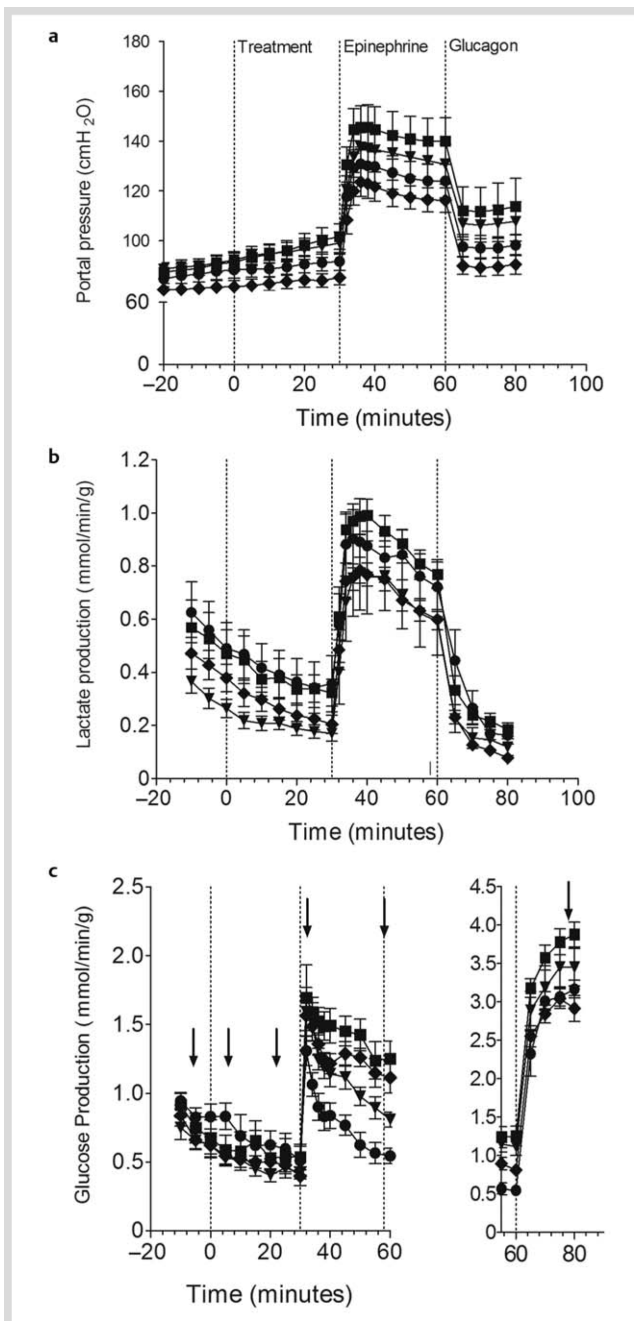

Fig. 1 Attenuation of HGO by DCI. Isolated rat livers (12–15 g) were perfused with plasma-free buffer containing 5 mM glucose (vehicle) in a non-recirculating set-up. The perfusion protocol comprised a 40 min pre-equilibration period followed by an infusion for 30 min with either buffer (■), 200 µM *myo*-inositol, (♦), 200 µM D-*chiro*-inositol (▼), or 1 nM insulin (●). Within the background of each of these experimental groups, an epinephrine co-infusion (50 nM) was commenced at 30 min to demonstrate organ viability and maintained until 60 min. Glucose, lactate, and portal pressure were monitored continuously. Livers were excluded from the analyses if they were unresponsive to the final glucagon stimulation at 60 min. All values are expressed as mean ± SEM. Statistical significance for each of the experimental groups between 32–60 min was assessed by fitting a mixed model analysis to the data using Residual Maximum Likelihood (REML) allowing for correlated errors between repeated observations on the same animal. Tukey-Kramer adjusted p-values were computed for differences between (least squares) means. Mean glucose ± SEM outputs at 60 min for each of the groups were 1.25 ± 0.13 µmol/min/g (n = 9) (buffer), 1.11 ± 0.11 µmol/min/g (n = 8) (*myo*-inositol), 0.81 ± 0.05 µmol/min/g (n = 10) (DCI), and 0.54 ± 0.06 µmol/min/g (n = 8) (insulin), respectively.

tizing actions in PCOS [11]. While putative DCI-containing mediators have been identified in crude liver lysates [1], the significance of the liver to the antidiabetic actions of DCI has not been investigated. The aim of the present study was to therefore investigate whether DCI evoked insulin-like effects on HGO using an isolated liver perfusion system.

Methods

Animals and reagents

This study was approved by the University of Auckland Animal Ethics Committee. Male Wistar rats (220–250 g) were maintained on a 12-h day/night cycle with water and food ad libitum (Harlan Teklad 2018 diet, Madison, WI, USA). BDH Chemicals were used unless otherwise stated. MOPS was procured from AppliChem and fatty acid free bovine serum albumin (BSA) was obtained from ICPBio, New Zealand (#APFF-010). All buffers were filtered using cellulose acetate filters (0.45 µm).

Isolated liver perfusion

Under general anaesthesia (isoflurane 2–5 %; 2 l/min O_2 via nasal cone) nonfasted rats (220–250 g) underwent laparotomy between 8:00–9:00 AM. The liver was removed and then perfused (non-recirculating mode) in a manner similar to that previously reported [12]. For the isolation of livers, the portal vein was cannulated in situ and the atria cut to allow the liver to be perfused initially with 20 ml of perfusion media comprising 128 mM NaCl, 23.9 mM MOPS, 6 mM KCl, 1.18 mM $MgSO_4 \cdot 7H_2O$, 1.29 mM $CaCl_2$, 0.2 % BSA (FFA), 5 mM glucose, pH 7.4. The in situ liver was then perfused at 2 ml/min while the vena cava was cannulated and then the liver was excised. The liver was weighed and perfused at a rate of $2\,ml \cdot g^{-1} \cdot min^{-1}$ with oxygenated perfusion media (buffer exposed to O_2: 95 %; CO_2: 5 %; 37 °C).

Results and Discussion

Our experiments were performed with livers in the fed state and in the absence of gluconeogenic substrates in the perfusion buffer. DCI (Industrial Research Limited, Lower Hutt, New Zealand), *myo*-inositol (Sigma-Aldrich), and insulin were each separately investigated with regard to their ability to attenuate epinephrine-stimulated HGO (**Fig. 1**). Prior to epinephrine co-infusion at 30 min, livers were first perfused with buffer during an equilibration period followed by addition of either buffer, insulin (1 nM), DCI (200 µM), or *myo*-inositol (200 µM) at t = 0 min. While there was some divergence in the baseline values, the addition of epinephrine nevertheless evoked similar increases in both portal pressure (**Fig. 1a**) and lactate output (**Fig. 1b**) across all the experimental groups.

In the buffer perfused livers (n = 9), epinephrine co-infusion at 30 min evoked an acute (within 2 min) approximately 3-fold increase in HGO that was largely sustained throughout the following 30-min period (squares, **Fig. 1c**). This increase in HGO evoked by epinephrine was attenuated between 32 and 60 min in livers co-infused with insulin (circles) (n = 10) ($p < 0.0001$). All livers were viable over the period of the perfusion as indicated by their responsiveness to glucagon, which was added to the buffer at 60 min (**Fig. 1c**). Despite an apparent reduction in HGO between 32–40 min, *myo*-inositol perfusion did not significantly alter HGO over the 30–60-min period as compared to the

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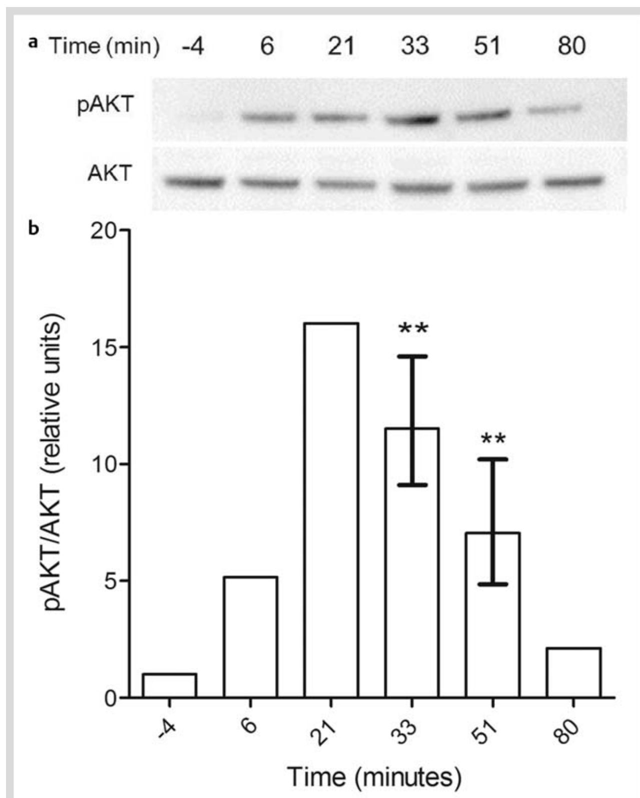

Fig. 2 Time course activation of Akt by insulin. **a** Western blot from one perfusion experiment with insulin perfusion. Liver lobes were excised at the times indicated and homogenised (OMNI, model TH220) in 0.8 ml of homogenisation lysis buffer containing phosphatase inhibitor (PhosSTOP phosphatase inhibitor cocktail tablets, Roche) and protease inhibitor (Complete protease inhibitor cocktail tablets, Roche). Samples were then centrifuged at 13 200 *g* for 20 min at 4 °C and the supernatants stored at − 80 °C before analysis. Following SDS/PAGE, gels were transferred onto PVDF membranes and analysed first for pAkt with a pS473Akt antibody [1:1 000 (#9271), Cell signalling technology]. Membranes were then stripped and reprobed for total Akt using an Akt antibody [1:1 000 (#9272), Cell Signalling Technology]. **b** Quantification of pS473Akt/Akt content. Data are shown as combined data from 2 independent perfusion experiments with insulin. Protein band intensities were quantified and normalised to the baseline time at − 4 min, which was prior to the addition of insulin to the perfusate. Data shown as geometric mean with 95 % confidence intervals shown only for those time points where the relative abundance of pAkt to Akt was significantly greater than 1 (based on one-sample *t*-test on log-transformed data). ** $p < 0.01$ vs. − 4 min.

control buffer perfusion (diamonds). HGO in the *myo*-inositol perfused livers was, however, elevated compared to insulin-perfused livers over the 30–60-min period ($p = 0.0035$).

Epinephrine-stimulated HGO in DCI-perfused livers ($n = 10$) was attenuated over the 32–60 min period to a degree intermediate between the buffer control ($p = 0.05$) and insulin perfusions ($p = 0.04$) (inverted triangles). After 60 min, both insulin and DCI reduced HGO by 57 % and 35 %, respectively, as compared to the buffer control. Thus, DCI acted as an insulin-mimetic in liver to reduce epinephrine-stimulated HGO. This reduction in HGO proceeded independently of insulin and was specific to the DCI stereoisomer as an identical concentration of *myo*-inositol (200 µM) had no effect on HGO.

We next investigated whether the insulin-mimetic effect evoked by DCI was mediated through the insulin-signalling pathway. Additional liver perfusions were performed for each of the buffer only, insulin, DCI, and *myo*-inositol experimental groups. The time course and procedures of these experiments were identical to the perfusion experiments described above (**Fig. 1**). During each perfusion, small samples of liver tissue were repeatedly isolated from various peripheral lobe locations (shown as arrows in **Fig. 1c**). These excisions were performed using a small tightly applied purse string suture to prevent subsequent leakage from the biopsy site. The excised liver sample was snap-frozen in liquid nitrogen for subsequent western blot analyses. There was no appreciable impact on liver perfusion pressures, or lactate production by removal of these small samples (data not shown).

For insulin-perfused livers, western blotting analyses showed activation of Akt (**Fig. 2a**). Compared to the baseline time point at − 4 min, which was prior to the addition of insulin at t = 0 min, pAkt/Akt content increased by approximately 16-fold after 21 min. Coincident with the addition of 50 nM epinephrine at 30 min, pAkt content decreased progressively thereafter (**Fig. 2b**). We found no evidence for activation of Akt in livers perfused either with buffer, DCI, or *myo*-inositol (data not shown). We also found no evidence for changes in the phosphorylation states of glycogen synthase-3 (GSK-3), AMP-activated protein kinase (AMPK), acetyl CoA carboxylase (ACC), and glycogen synthase across all the 4 groups (data not shown). These results suggested that the observed decrease in epinephrine-stimulated HGO evoked by DCI was not mediated via activation of Akt.

These findings show for the first time that DCI can attenuate epinephrine-stimulated HGO in the liver. Circulating levels of DCI following DCI administration in rat models of diabetes have not been reported so direct comparison with the concentration of DCI (200 µM) used in our perfusion experiments is not possible. In nondiabetic rats endogenous circulating plasma levels of DCI are maintained within the low micromolar range and derived almost exclusively from standard chow diets, which are comparatively rich in DCI content [13]. It is less clear what circulating DCI levels are in diabetic rats as renal excretion of DCI is selectively increased compared to the nondiabetic state [14]. Nevertheless, our findings suggest that DCI-mediated inhibition of HGO could contribute to DCI's antidiabetic effects.

In addition, the glucose-lowering effects were specific to DCI as the corresponding stereoisomer, *myo*-inositol, had no overall effect on HGO. This finding is consistent with studies showing that the insulin-mimetic properties are confined to DCI [1]. Also, attenuation of HGO by DCI occurred in the absence of insulin and Akt activation suggesting that circulating DCI could exert a tonic effect on HGO in vivo.

DCI-containing mediators were identified originally from crude liver lysates and one such mediator, comprising a pinitol galactosamine pseudo disaccharide (INS-2), reportedly promotes nonoxidative and oxidative glucose disposal in skeletal muscle [1]. These actions by INS-2 may be mediated through stimulation of protein phosphatase 2Cα (PP2Cα) and pyruvate dehydrogenase phosphatase (PDHP) activities, respectively [1].

Inhibition of HGO by DCI provides indirect support for the existence of mediators, such as INS-2, and their action on metabolic targets in liver that regulate glycogen metabolism. DCI is readily transported into liver cells through an inositol transporter so it is possible that DCI is internalised and converted directly into functionally active DCI-containing mediators, such as INS-2. Further investigation of the protein targets modified by DCI-containing mediators in liver could therefore provide new avenues to regulate hyperglycaemia in diabetes with wider

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implications for the associated insulin-sensitizing effects observed in PCOS.

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Conflict of Interest

The authors have no conflict of interest.

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References

1 *Larner J, Brautigan DL, Thorner MO.* Mol Med 2010; 16: 543–552
2 *Fonteles MC, Almeida MQ, Larner J.* Horm Metab Res 2000; 32: 129–132
3 *Nestler JE, Jakubowicz DJ, Reamer P, Gunn RD, Allan G.* N Engl J Med 1999; 340: 1314–1320
4 *Kennington AS, Hill CR, Craig J, Bogardus C, Raz I, Ortmeyer HK, Hansen BC, Romero G, Larner J.* N Engl J Med 1990; 323: 373–378
5 *Asplin I, Galasko G, Larner J.* Proc Natl Acad Sci USA 1993; 90: 5924–5928
6 *Ostlund RE Jr, McGill JB, Herskowitz I, Kipnis DM, Santiago JV, Sherman WR.* Proc Natl Acad Sci USA 1993; 90: 9988–9992
7 *Baillargeon JP, Diamanti-Kandarakis E, Ostlund RE Jr, Apridonidze T, Iuorno MJ, Nestler JE.* Diabetes Care 2006; 29: 300–305
8 *Nascimento NR, Lessa LM, Kerntopf MR, Sousa CM, Alves RS, Queiroz MG, Price J, Heimark DB, Larner J, Du X, Brownlee M, Gow A, Davis C, Fonteles MC.* Proc Natl Acad Sci USA 2006; 103: 218–223
9 *Farias VX, Macedo FH, Oquendo MB, Tome AR, Bao SN, Cintra DO, Santos CF, Albuquerque AA, Heimark DB, Larner J, Fonteles MC, Leal-Cardoso JH, Nascimento NR.* Diabetes Obes Metab 2011; 13: 243–250
10 *Leonard BL, Watson RN, Loomes KM, Phillips AR, Cooper GJ.* Acta Diabetol 2005; 42: 162–170
11 *Home PD, Pacini G.* Diabetes Obes Metab 2008; 10: 699–718
12 *Englisch R, Wurzinger R, Furnsinn C, Schneider B, Frisch H, Waldhausl W, Graf J, Roden M.* Biochem J 2000; 351: 39–45
13 *Lin X, Ma L, Gopalan C, Ostlund RE.* Brit J Nut 2009; 102: 1426–1434
14 *Kawa JM, Przybylski R, Taylor CG.* Exp Biol Med (Maywood) 2003; 228: 907–914

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