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Dissection of mechanisms that account for imidazoline-induced lowering of blood glucose in mice

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ABSTRACT

Multiple mechanisms have been suggested to be responsible for the insulinotropic and blood glucose lowering effects of imidazoline compounds. This study was to unravel which mechanism predominantly accounts for glucose lowering by the prototypical imidazolines idazoxan and phentolamine. To this end, an α_2-adrenoceptor agonist (UK14,304) and a K_{ATP} channel opener (diazoxide) were used to inhibit insulin release from isolated perifused mouse islets and to induce hyperglycaemia in conscious mice. Potentials of idazoxan and phentolamine to counteract these effects were examined in a comparative manner. In perifused islets, idazoxan increased insulin release only in the presence of the α_2-agonist, whereas phentolamine strongly counteracted both inhibitors of insulin release. *In vivo*, a lower dose of idazoxan was necessary to ameliorate hyperglycaemia induced by the α_2-agonist than by the K_{ATP} channel opener, indicating α_{2A}-antagonism as the predominant mechanism of action (decrease in incremental area under the glucose curve induced by 0.1 mg/kg idazoxan: under diazoxide, $-3 \pm 7\%$, vs. under UK14,304, $-34 \pm 9\%$, $P < 0.02$). In contrast, identical doses of phentolamine were required to counteract hyperglycaemia induced by the two inhibitors of insulin release, implicating involvement of another mechanism beside α_{2A}-antagonism (2 mg/kg phentolamine: diazoxide, $-11 \pm 8\%$, vs. UK14,304, $-15 \pm 9\%$, ns; 4 mg/kg phentolamine: diazoxide, $-48 \pm 6\%$, vs. UK14,304, $-48 \pm 8\%$, ns). The results show that imidazolines can lower blood glucose via more than one mechanism of action, with the relative contributions of the mechanisms varying considerably between individual compounds. Dissection of the involved mechanisms could help to develop imidazoline drugs for the treatment of type 2 diabetes.

1. Introduction

Since decades imidazoline compounds are known to exert insulinotropic and blood glucose lowering effects. While their antihyperglycaemic but not hypoglycaemic characteristics suggest advantages over prevalently prescribed sulfonylureas and glinides, uncertainties about their mechanism(s) of action persist and hamper the development of imidazoline drugs for clinical use.

Actions of the prototypical imidazoline phentolamine were initially explained by antagonism at α-adrenoceptors, with the increase in insulin release attributed to relief of β-cells from an increased sympathetic tone that prevails in diabetic patients (Buse et al., 1970; Robertson et al., 1976; Robertson and Porte, 1973). Although pancreatic β-cells express the adrenoceptor subtype α_{2A} which mediates inhibition of insulin secretion (Angel et al., 1990; Fagerholm et al., 2004, 2011), this early paradigm was put into doubt by the observation that the potent α_2-antagonistic imidazoline idazoxan failed to affect circulating glucose and insulin in normal and diabetic subjects (Östenson et al., 1988). Subsequent systematic investigations showed that the insulinotropic activities of individual imidazolines *in vitro* deviated markedly from their α_2-antagonistic properties, which included that idazoxan fell short of phentolamine´s impact on pancreatic β-cells in several experimental settings (Chan and Morgan, 1990; Östenson et al., 1989; Rustenbeck et al., 1999; Schulz and Hasselblatt, 1989; Shepherd et al., 1996). Together with the finding of non-imidazoline α-antagonists devoid of insulinotropic activity (Schulz and Hasselblatt, 1988, 1989), this resulted in the idea that insulin release was mediated by an imidazoline-preferring site, sometimes referred to as the "I_3-receptor" (Efendic et al., 2002; Morgan and Chan, 2001).

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The finding was made that imidazolines can block K_{ATP} channels on β-cells, apparently due to interaction with the pore-forming subunit Kir6.2 (regarded as the correlate of the I_3-receptor) (Hatlapatka et al., 2009; Monks et al., 1999; Morgan and Chan, 2001; Proks and Ashcroft, 1997; Rustenbeck et al., 1995, 1999; Szollosi et al., 2010). This provided a plausible alternative explanation for promotion of insulin release, as further supported by evidence that idazoxan is a less effective K_{ATP} channel blocker than the insulinotropic imidazolines phentolamine and efaroxan (Chan and Morgan, 1990; Östenson et al., 1989; Rustenbeck et al., 1999; Shepherd et al., 1996). But again, the whole matter became complicated by evidence for another site of action distal to Ca^{2+} influx into the β-cell, which some regarded as the truly important mechanism of insulinotropic action (Chan et al., 2001; Efendic et al., 2002; Meidute-Abaraviciene et al., 2009; Sharoyko et al., 2007; Zaitsev et al., 1996).

More recently, evidence arose that variations in or near the gene encoding the α_{2A}-adrenoceptor (ADRA2A) may pre-dispose individuals to type 2 diabetes, which led to speculations that α_{2A}-antagonists could be a treatment tailored to those carrying the at-risk mutations (Gribble, 2010; Liggett, 2009; Rosengren et al., 2010; Wess, 2010). Together with a revival of the previously discarded idea that α_{2A}-antagonism could be relevant to the metabolic effects of imidazolines (Fagerholm et al., 2008), these findings inspired us to re-evaluate the pharmacological properties of the imidazoline efaroxan, a particularly strong α_2-antagonist. Our investigations in mice resulted in the first conclusive demonstration of an imidazoline, which is able to trigger antihyperglycaemic effects via at least two divergent mechanisms. The outcome suggested that these were α_2-antagonism on one hand and closure of K_{ATP}-channels on the other, whereby glucose lowering via the latter mechanism required much higher doses of the drug (Lehner et al., 2012). Exploiting experimental protocols used to dissect the mechanisms of efaroxan action, the present study aimed to ultimately explain divergency between the pharmacological profiles of two classic imidazolines, idazoxan and phentolamine. While extensive research has already clarified that imidazolines can augment insulin release *in vitro* via more than one mechanism, the crucial question about respective contributions of these mechanisms to glucose lowering *in vivo* remained unanswered. Hence, it was our particular ambition to define, if and to what extent idazoxan and phentolamine address different mechanisms to lower blood glucose *in vivo*.

2. Materials and methods

2.1. Mice

Male C57BL/6J mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and were used within an age range of 12 to 26 weeks. Mice were kept at constant room temperature and an artificial 12 h-light/12 h-dark cycle. Unless stated otherwise, they had access to conventional pellet diet and tap water *ad libitum*. The study protocol was approved by the Austrian Federal Ministry for Science and Research and followed all principles of good laboratory animal care.

2.2. Islet perifusion

Procedures of islet perifusion have been described before (Lehner et al., 2012). In short, mice in the fed state were anaesthetised and killed by cervical dislocation. 3 ml collagenase solution was then injected into the common bile duct, which was clamped at the papilla vateri (1 g/l Collagenase NB 8 Broad Range from Serva, Heidelberg, Germany; dissolved in ice-cold KRB=Krebs–Ringer buffer containing 0.2% BSA and 5 mmol/l HEPES; pH adjusted with NaOH to 7.35). Pancreatic tissue as distended by this injection was incubated at 37 °C for collagenase digestion, which was stopped after 13 min by two cycles of centrifugation and resuspension in ice-cold KRB additionally supplemented with a glucose concentration as used during the initial period of the subsequent perifusion experiment (5 or 7 mmol/l, see below).

Fifty intact islets were collected under the microscope, transferred to a custom-made perifusion chamber, and continuously perifused with KRB (37 °C; 1 ml/min) containing a DMSO concentration that was identical in experiments subjected to statistical comparison ($\leq$0.25%vol/vol). The protocols started with a 60 min equilibration period (0–60 min), which was followed by 60 min (60–120 min) of exposure to vehicle, idazoxan, phentolamine, or gliclazide at concentrations as indicated in the results section (all from Sigma, St. Louis, MO, USA). In experiments under such standard conditions, the glucose concentration was 5 mmol/l during the first 90 min followed by 10 mmol/l during the last 30 min. In most experiments, however, an inhibitor of insulin secretion was added to the medium during the entire perifusion period (250 μmol/l of the K_{ATP} channel opener diazoxide, or 1 μmol/l of the α_2–adrenergic agonist UK14,304; both from Sigma), and an initial glucose concentration of 7 mmol/l was raised to 20 mmol/l during the final 30 min (i.e., 90–120 min). At 40, 50, 60, 62.5, 65, 67.5, 70, 75, 80, 90, 92.5, 95, 97.5, 100, 105, 110, and 120 min, effluent was collected over 1 min and frozen for the later measurement of insulin (Sensitive Rat Insulin RIA Kit, Millipore, Billerica, MA, USA).

2.3. Glucose tolerance test and hyperglycaemia induced by inhibitors of insulin release

All experiments performed *in vivo* followed protocols as employed previously (Lehner et al., 2012). Individual mice were repeatedly used for up to 8 *in vivo*-tests, which were separated by washout/recovery periods of at least 7 days. To account for day-to-day variation in blood glucose as typically seen in mice, statistical analysis and interpretation relied only on comparison of data sets obtained within the same experimental run, which implicates that a separate control group (n=8 or 9) was studied on each working day.

2.3.1. Glucose tolerance test

Mice were fasted for 10 h and the tip of the tail was pricked with a needle for the duplicate measurement of blood glucose with a portable glucose metre (OneTouch, LifeScan, Milpitas, CA, USA). Immediately thereafter, vehicle (0.5% sodium carboxymethylcellulose, CMC; 5 ml/kg), or the indicated dose of idazoxan, phentolamine, or gliclazide was orally administered by gavage. 45 min later, mice received an aqueous glucose solution (50% wt/vol; 3 g/kg) by gavage. Further glucose measurements were made immediately before (0 min) and 30, 60, 90, 120, and 150 min after glucose administration.

2.3.2. Pharmacologically induced hyperglycaemia

To study the interaction of the test compounds with pharmacological inhibitors of insulin secretion, endogenous hyperglycaemia was induced with diazoxide (250 mg/kg) or UK14,304 (100 μg/kg). Diazoxide was admixed to the respective gavage solution (6 ml/kg) containing idazoxan, phentolamine, or gliclazide, whereas UK14,304 or its vehicle (saline containing 7% DMSO; 5 ml/kg) was injected intraperitoneally immediately after the gavage. Blood glucose was measured immediately before the treatment (0 min) as well as 30, 60, 90, 120, 150, and 180 min thereafter.