Hepoxilins raise circulating insulin levels in vivo

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Abstract We have demonstrated over a decade ago that hepoxilins cause the release of insulin from isolated pancreatic islets of Langerhans in vitro. However, no studies are available so far to indicate whether these compounds are active in vivo. The present study is the first to our knowledge which demonstrates that hepoxilins administered intra-arterially in the anaesthetized rat cause the release of insulin in the circulation. This release is dependent on the glucose status of the rat. Hence, animals fasted overnight do not respond to hepoxilin administration, while animals that have had free access to food respond to hepoxilins with a rise in insulin concentrations in blood. The hepoxilin effect is rapid and varies with different hepoxilins, the most potent of which is hepoxilin A_3 (HxA_3) (both the 8*S* and the 8*R* enantiomers). Administration of 100 μg HxA_3 produces a rise in blood insulin equivalent to that caused by the administration of 5 mg glucose. In view of earlier evidence showing that these compounds cause a rise in intracellular calcium levels in vitro at a <1 μg/ml concentration through a receptor-mediated mechanism, we speculate that the actions of hepoxilins in causing the release of insulin from the pancreas may be due to alterations in calcium levels within the β-cell. We believe that hepoxilins may represent new lead compounds as therapeutics in type II diabetes mellitus.

Key words: Hepoxilin; Insulin; Diabetes mellitus; Calcium

1. Introduction

Hepoxilins were discovered in the early 1980s [1,2]. They are lipidic compounds derived from arachidonic acid via the 12-lipoxygenase pathway [3,4]. Hence, 12(*S*)-HPETE, the initial product of 12(*S*)-lipoxygenase, is enzymatically transformed into two hydroxy-epoxide products named hepoxilin A_3 (HxA_3) and hepoxilin B_3 (HxB_3) [5]. The in vivo formation of HxA_3 after administration of arachidonic acid in the rat has been reported [2]. These compounds are biologically unstable through enzymatic pathways known to catabolize them [6]. Early studies in the rat showed that HxA_3 was capable of effecting the release of insulin from isolated perifused islets of Langerhans in vitro [7]. Additional studies using single cells showed that HxA_3 caused a rise in intracellular calcium that was independent of extracellular calcium, indicating that hepoxilin caused the release of calcium from intracellular calcium stores [8]. Calcium release is mediated by a hepoxilin-specific receptor present in the cell [9–11]. The purpose of this study was to investigate whether the native hepoxilins (A and B type) cause the release of insulin in vivo. No such studies have been reported.

2. Materials and methods

2.1. Materials

HxA_3 (8*R* and 8*S* epimers) and HxB_3 (10*R* and 10*S* epimers) were chemically synthesized as described previously [12]. Male Wistar rats (200–220 g, body weight) were purchased from Charles River (St. Constant, Que., Canada). They were maintained either without food overnight or with regular food chow ad lib, with unlimited access to water. On the day of the experiment, the animals were anaesthetized with inactin (100 mg/kg, intraperitoneal; BYK-Gulden, Constanz, Germany). The trachea and left carotid artery were cannulated for respiration and blood sampling, respectively. Body temperature was maintained at 37°C by placing animals on a thermostatically controlled heating box. The animals were lightly heparinized to prevent blood coagulation during the experiment.

2.2. Experimental protocol

Two hours were allowed after the completion of surgery for the animals to stabilize. Blood samples were withdrawn (300 μl) at the following time points: −10 min, −5 min and 30 s, 2, 5, 10, 30, 60 and 120 min after administration of the test compound. Fluid replacement equivalent to the blood withdrawn was carried out by administration of heparinized saline. Groups of animals (five/group) were set up for each test hepoxilin. The hepoxilins were made up in ethanol so that the test dose (100 μg/animal) was injected with a Hamilton syringe in 10 μl ethanol through a plastic Y-injector while 300 μl saline was being injected. Hence, the ethanol was carried into the animal while being diluted with saline. This was important as hepoxilins may precipitate in saline solution alone. Blood was collected in a plastic syringe at the designated times and added to Eppendorf tubes. After 1 h, the samples were centrifuged and the serum was collected and stored at −70°C until assayed for insulin by RIA. Glucose concentrations were measured in the serum samples with a glucosimeter. A control group was studied in which ethanol alone (in the absence of hepoxilins) was administered. In another group, glucose was made up at different concentrations in water (1, 10, 25 and 50 mg/100 μl) and 100 μl of this solution was administered while heparinized saline was being flushed into the Y-injector as in the test groups. This was carried out to attempt to titrate the insulin changes by the hepoxilins relative to the changes observed with glucose administration.

2.3. Measurements

Insulin was measured by a double antibody RIA [13]. Rat insulin kits were purchased from LINCO (St. Louis, MO, USA) and the method was modified to use 20 μl instead of 100 μl sample volume. The detection limit was 18 pmol/l. Glucose was measured by the oxygen consumption method using 10 μl sample volume [14]. The limit of detection was 0.3 mmol/l.

3. Results

Administration of a bolus dose of glucose intra-arterially causes a rapid immediate increase in insulin concentration in the circulation. Fig. 1 shows time-related changes observed after the administration of 50 mg glucose (200 mg/kg) in the

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Abbreviations: HxA_3, hepoxilin A_3

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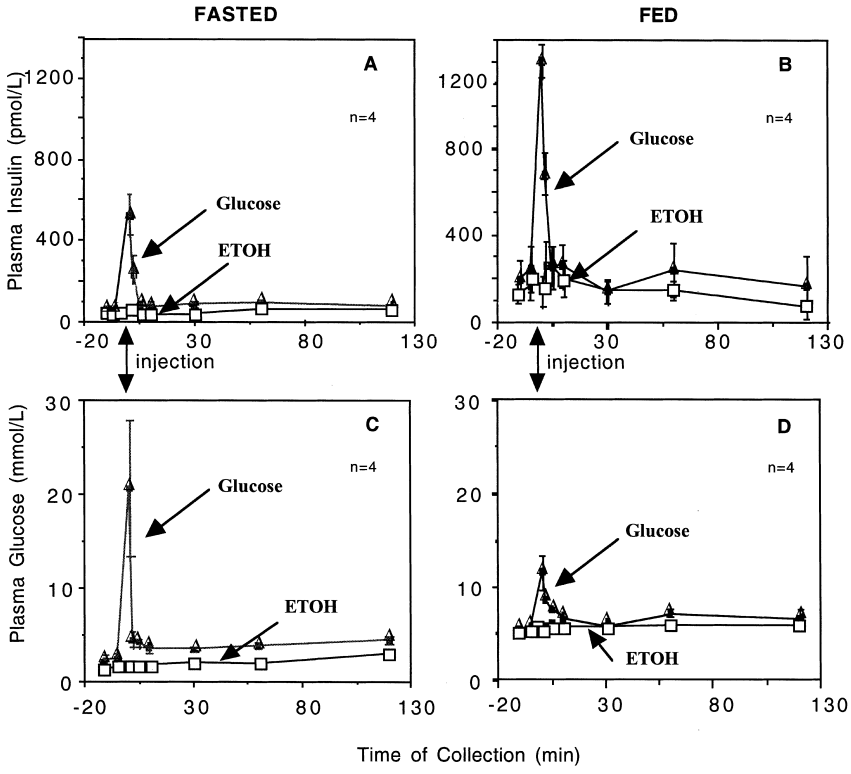

Fig. 1. Temporal changes in insulin and glucose concentrations in blood of inactin-anaesthetized rats in which bolus amounts of glucose (50 mg/rat = 200 mg/kg) or 10 μl ethanol (the vehicle for the hepoxilins) were administered through a carotid cannula. Rats had either no access ('fasted') or free access ('fed') to food the evening before the experiment. The compounds were injected during the co-injection of 300 μl of saline in a plastic Y-injector attached to the carotid cannula. At the times indicated, i.e. −10, −5 min pre-injection and 30 s, 2, 5, 10, 30 and 60 min post-injection of hepoxilin (see arrow at the point of injection), 300 μl of blood was withdrawn followed by fluid replacement with saline. Blood samples were allowed to clot and the serum was stored until analysis for insulin and glucose was performed as indicated in the Section 2.

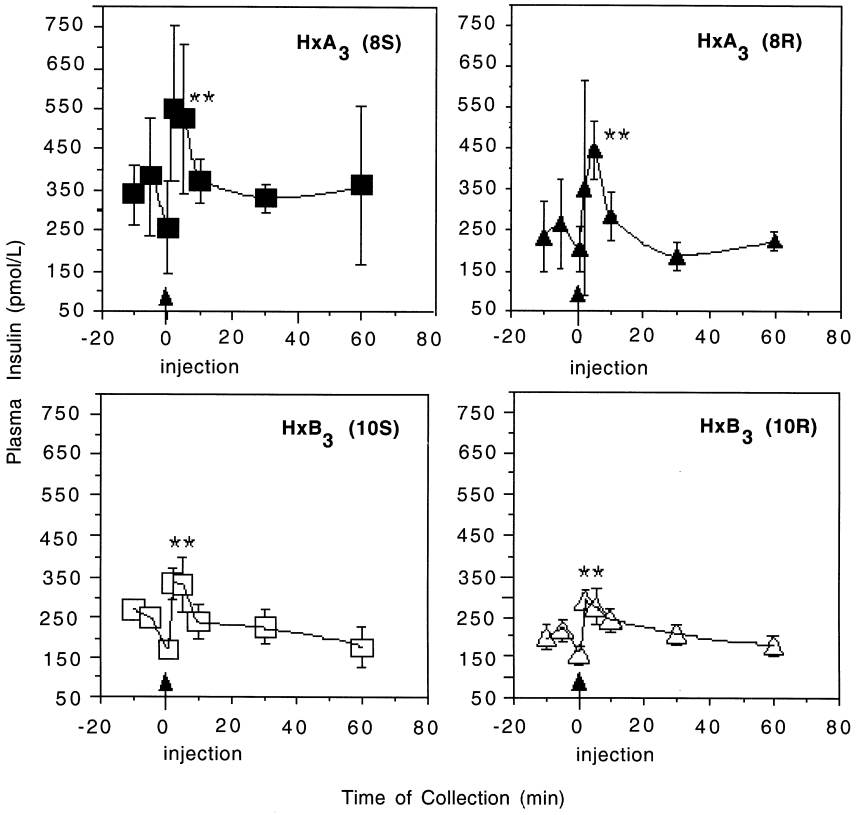

Fig. 2. Temporal changes in insulin concentrations in blood of inactin-anaesthetized 'fed' rats in which bolus amounts of the four hepoxilins indicated at a dose of 100 μg/rat in 10 μl ethanol were administered intra-arterially as described in Fig. 1. $^{**}P < 0.05$.

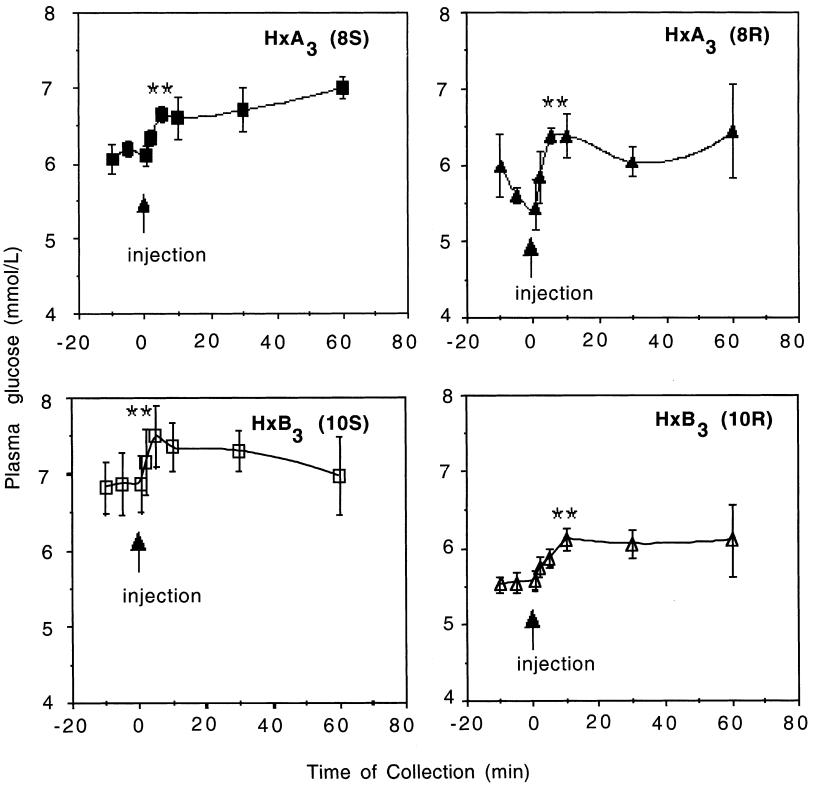

Fig. 3. Temporal measurements in glucose concentrations in blood of inactin-anaesthetized rats from the experiments shown in Fig. 2. $^{**}P < 0.05$.

adult rat. Also shown are the glucose concentrations in blood of these animals. A significant difference was observed in the insulin blood concentration depending on the nutritional state of the animal, i.e. animals fasted overnight responded with a lesser release of insulin in the blood than animals that were allowed free access to food (Fig. 1). The glucose-evoked release of insulin was dose dependent (not shown).

Initially, we investigated actions of hepoxilins in the fasted rat but observed no change in insulin blood levels over the pre-hepoxilin levels (not shown). In view of the experiments with glucose administration where the fed animals were more responsive in releasing insulin, we tested the hepoxilins in the fed animal. Indeed, a rise in blood insulin concentrations was observed after hepoxilin administration. Fig. 2 compares the actions of four hepoxilins, i.e. the 8*S*/8*R* epimers of HxA_3 and the 10*S*/10*R* epimers of HxB_3 at the bolus dose of 100 μg/animal. Two observations can be noted. First, there is a difference between the extent of insulin release by each of the four compounds, the 8*S* epimer of HxA_3 being the most potent while the 10*R* epimer of HxB_3 seems to have little effect. Secondly, while the insulin release caused by glucose administration peaked at the 30 s time point after intra-arterial injection, the hepoxilins peaked at the 2 min sampling point, suggesting a delay in hepoxilin uptake by the pancreas or that the hepoxilins act via activation of some other messenger to cause the release of insulin. Neither of the hepoxilins caused a pronounced change in the glucose concentrations in blood although the small changes observed were significant (Fig. 3).

4. Discussion

Previous studies have shown that HxA_3 causes the release of insulin from isolated incubated islets [15] or perifused islets of Langerhans [7] in vitro. This was confirmed by other investigators [16]. Hepoxilins also cause the release of calcium from intracellular stores [17] through a hepoxilin-specific binding protein [9–11] present in neutrophils and presumably other cell types which respond to the hepoxilins. Indeed, isolated β-cells respond to hepoxilins with a release of insulin (C.R. Pace-Asciak, unpublished observations). Hence, the hepoxilin-evoked release of insulin from the β-cell may be mediated through their actions on calcium.

As hepoxilins are lipidic in nature and rather unstable biologically (through catabolism) and chemically (through epoxide ring opening) [18], we wished to know whether hepoxilins could effect insulin release in vivo, as this could be severely hindered by limited uptake by the pancreas. The present results demonstrate that the native hepoxilins, especially those of the A series, cause a rise in circulating insulin. We found this release to be affected by the glucose status of the rat, being insensitive to hepoxilins in the fasted state but significantly active in animals that were allowed free access to food. Preliminary unpublished observations with tritium-labelled hepoxilin indicated that approximately 2% of the injected radiolabel was observed in the pancreas at 2 min post-injection, the peak period of insulin release (Fig. 2), indicating that the effective potency of the compounds is considerably greater than the dose used (i.e. at about 2 μg/rat), assuming that

most of this radiolabel is accounted for by the native compound and not to inactive catabolites. Hence, hepoxilins possess considerable biological potency.

These observations offer new insights into novel lead compounds involved in the release of insulin in vivo. Because the native hepoxilins are rather unstable, we are pursuing ways of stabilizing their structure chemically and biologically with an aim at developing novel therapeutic agents of potential use in type II diabetes mellitus.

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