

## $\beta$ -Cell $\alpha$ -ketoglutarate hydroxylases may acutely participate in insulin secretion

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### Abstract

The presence of Fe(II)  $\alpha$ -ketoglutarate hydroxylases in rat and human pancreatic islets and INS-1 832/13 cells was demonstrated with the reverse transcriptase polymerase chain reaction (PHD1, 2, and 3; lysyl hydroxylases 1, 2, and 3; and phytanoyl-coenzyme A hydroxylase were seen) and/or immunoblotting (high levels of proline hydroxylase P4H $\alpha$ 1, PHD2, and PHD4 and low levels of PHD2 and PHD3 in human islets, and high levels of PHD2 in rat islets and INS-1 cells were seen). Prolyl hydroxylase enzyme activity in INS-1 832/13 cells was purified with polyproline affinity chromatography. Inhibitors of  $\alpha$ -ketoglutarate hydroxylases lowered glucose-induced and leucine-plus-glutamine-induced insulin release in rat pancreatic islets, suggesting that there may be acute unknown effects of  $\alpha$ -ketoglutarate hydroxylases in insulin secretion. It is possible that an increase in mitochondrially generated  $\alpha$ -ketoglutarate derived from insulin secretagogue carbon and translocated to the cytosol may be part of the signal for insulin secretion.

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### 1. Introduction

Fuel secretagogues stimulate insulin secretion by their metabolism in  $\beta$ -cell mitochondria. Not only does this produce adenosine triphosphate that powers cellular processes, but also mitochondria synthesize citric acid cycle intermediates (anaplerosis) that are exported from mitochondria to the cytosol, where they are converted to metabolites that have signaling and supporting roles in insulin exocytosis [1]. Roles have been proposed for several citric acid cycle intermediates, including malate and citrate that may participate in shuttles of equivalents of reduced and/or oxidized pyridine nucleotides across the inner mitochondrial membrane [2–8], or the export of short-chain acyl groups for synthesis of short-chain acyl-coenzyme As (CoAs) in the cytosol [9–13], or the regulation of cellular metabolic oscillations [14].  $\alpha$ -Ketoglutarate formed in the mitochondria can be transported across the inner mitochondrial membrane to the cytosol in exchange for malate [1]. However, except for the participation of  $\alpha$ -ketoglutarate in the malate aspartate shuttle [2], no specific extramitochondrial role for  $\alpha$ -ketoglutarate in insulin secretion has been previously

mentioned. In the current study, we investigated whether the  $\alpha$ -ketoglutarate hydroxylase family of enzymes might have a role in insulin secretion. Fe(II)  $\alpha$ -ketoglutarate-dependent hydroxylases catalyze a diverse array of reactions in non-islet tissues [15]. Primary substrates include prolyl, lysyl, and aspartyl residues in proteins, as well as lipids. Oxidative decarboxylation of  $\alpha$ -ketoglutarate forms CO<sub>2</sub> plus succinate and leads to the generation of an oxoiron radical or other activated oxygen species that hydroxylate the primary substrate [15]. The current study shows that inhibitors of  $\alpha$ -ketoglutarate hydroxylases markedly decreased insulin release from pancreatic islets, possibly indicating that  $\alpha$ -ketoglutarate translocated from mitochondria is necessary for insulin secretion by serving as a substrate for these cytosolic hydroxylases. Reverse transcriptase polymerase chain reaction (RT-PCR) experiments indicated that transcripts for prolyl and lysyl hydroxylases and phytanoyl-CoA hydroxylases are present in human and rat pancreatic islets and INS-1 cells. In addition, we detected  $\alpha$ -ketoglutarate hydroxylase activity with endogenous protein substrates in INS-1 cell cytosol. Prolyl hydroxylase enzyme activity was identified in INS-1 cells by purifying the enzyme activity with polyproline affinity chromatography.

The rapid inhibition of insulin release by inhibitors of  $\alpha$ -ketoglutarate hydroxylases indicates that these hydroxylases

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have an acute influence on insulin secretion. Well-known actions of  $\alpha$ -ketoglutarate hydroxylases are in slower processes, including collagen formation and transcription factor activation [15]. Thus, the results may indicate that there are new and, as yet, unidentified substrates for  $\alpha$ -ketoglutarate hydroxylases in the pancreatic  $\beta$ -cell.

## 2. Experimental procedures

### 2.1. Materials

(Pro-pro-gly)<sub>10</sub> was from Peptides International, Louisville, KY. (Ile-lys-gly)<sub>3</sub> was synthesized by the University of Wisconsin Biotechnology Center. All other chemicals were from Sigma Chemical (St Louis, MO) in the highest purity available. Human pancreatic islets were from the Islet Isolation Core at Washington University School of Medicine, St Louis. INS-1 832/13 cells were from Chris Newgard [16].

### 2.2. General methods

Pancreatic islet isolation, insulin release studies, glucose oxidation studies, and subcellular fractionation of islets and INS-1 cells were done as previously described [3,17–19].

### 2.3. RT-PCR analysis

Total RNA from pancreatic islets and INS-1 cells was isolated with the Qiagen (Valencia, CA) RNeasy kit. The RNA (1–2  $\mu$ g) was reverse transcribed with the Ambion (Austin, TX) RETROscript kit in a volume of 20  $\mu$ L. Reverse transcription reaction mixture (1  $\mu$ L) was then added to 10  $\mu$ L of PCR reaction mixture, and amplification was performed with 1 U of Sigma REDtaq genomic DNA polymerase and 200  $\mu$ mol/L deoxyribonucleoside triphosphate with sets of forward and reverse primers designed from DNA sequences identified from the following GenBank accession numbers: for the rat hydroxylases—prolyl hydroxylases 1, 2, and 3: AY229997, AY228140, and NM\_019371; lysyl hydroxylases 1, 2, and 3: NM\_053827, AJ430861, and NM\_178101; phytanoyl-CoA hydroxylase: NM\_053674; and mouse aspartyl hydroxylase: NM\_133723; for the human hydroxylases—prolyl hydroxylases 1, 2, 3, and 4: NM\_053046.2, NM\_022051, NM\_022073.2, and NM\_177938; lysyl hydroxylases 1, 2, and 3: NM\_000302.2, NM\_182943.1, and NM\_001084.3; phytanoyl-CoA hydroxylase: IVM\_006214.2; and aspartyl hydroxylases: NM\_004318, NM\_032468.1, and NM\_032466.1. Primer sequences are available upon request.

### 2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot analyses

Total cellular protein or cytosol was boiled in Laemmli sample buffer and added in amounts equivalent to 50 pancreatic islets, or more islets as designated in individual legends, to 1.5-mm-thick 10% sodium dodecyl sulfate–polyacrylamide gels and electrophoresed in a Mini-Protein II gel apparatus (BioRad, Hercules, CA). Samples were

transferred to nitrocellulose membranes in a Mini Trans-Blot apparatus (BioRad), probed with antibodies and electrochemiluminescence reagents, and exposed to Kodak (Rochester, NY) BioMax MR film for 1 or 5 minutes. Antibodies were used at a 1:500 dilution and were raised against proteins with the following GenBank protein ID numbers: prolyl hydroxylase P4H $\alpha$ 1 (ICN Biomedicals, Costa Mesa, CA) (NP\_000908 [human] and NP\_742059 [rat]) and HIF prolyl hydroxylases (Novus Biologicals, Littleton, CO) HIF prolyl hydroxylase 1 (PHD1, EglN2) (NP\_444274 [human] and AA046039 [rat]), HIF PHD 2 (EglN1) (NP\_071334 [human] and AA034711 [rat]), HIF PHD 3 (EglN3) (NP\_071356 [human] and NP\_062244 [rat]), and HIF PHD 4 (NP\_80887 [human]).

### 2.5. Affinity chromatography of prolyl hydroxylase

Polyproline was coupled to agarose as previously described [20]. Poly-L-proline ( $M_r \approx 30\,000$ ) (36 mg) (Sigma Chemical catalog number p3886) was dissolved in 2 mL of 0.15 mol/L NaCl, 0.1 mol/L NaHCO<sub>3</sub> buffer, pH 9.3, and added to a suspension of 2.5 mL of CNBr-activated Sepharose 4B (250 mg dry weight) (Sigma). The mixture was gently rotated for 20 hours at 4°C, and then the gel was washed with 10 vol of 0.1 mol/L NaCl, 0.1 mol/L glycine, 10  $\mu$ mol/L dithiothreitol, and 10 mmol/L Tris-HCl buffer, pH 7.8, for 2 hours. The gel was kept in the same buffer for 20 hours before it was washed with column buffer and used for affinity chromatography.

About 0.5 mL of packed 832/13 INS-1 cells that were cultivated as monolayers in INS-1 medium [21] (RPMI 1640 tissue culture medium [the glucose concentration in this medium is 11.1 mmol/L] supplemented with 10% fetal bovine serum, 1 mmol/L pyruvate, 50  $\mu$ mol/L  $\beta$ -mercaptoethanol, and 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer [12,13]) and penicillin (100 U/mL) and streptomycin (100  $\mu$ g/mL) were washed 3 times in phosphate-buffered saline and homogenized in 1.2 mL of 0.1 mol/L NaCl, 0.1 mol/L glycine, and 0.01 mol/L Tris-HCl buffer, pH 7.8 (column buffer), containing 0.2% Triton X-100. The homogenate was centrifuged at 16 000g  $\times$  10 minutes, and the supernatant fraction was saved. The resulting pellet was rehomogenized in 0.6 mL of the same buffer solution and centrifuged, and the supernatant fractions were combined. The supernatant fraction volume (2 mL) was increased to 3.4 mL with buffer and applied to a (0.14 cm<sup>2</sup>  $\times$  3.5 cm) (bed volume, 0.5 mL) poly-L-proline Sepharose-4B column at a flow rate of 0.1 mL/min. The column flow through was collected and applied to the column a second time. The second column flow through was collected and saved. The column was then washed with 2 mL of buffer, and 1 mL of 5 mg/mL poly-L-proline ( $M_r$  8900) was applied to the column at a flow rate of 0.05 mL/min to elute the prolyl hydroxylase. Column fractions were concentrated 3- to 4-fold by centrifuging at 14 000g for 40 to 60 minutes in a Microcon YM-10 Centrifugal Filter Device (10 000 molecular weight cutoff) (Millipore).

2.6. Enzyme assays

$\alpha$ -Ketoglutarate hydroxylase activity was estimated in a final volume of 50  $\mu$ L reaction mixture containing 18 to 25  $\mu$ L of enzyme sample and 200  $\mu$ mol/L Tris(2-carboxyethyl) phosphine hydrochloride, 100  $\mu$ mol/L FeSO<sub>4</sub>, 2 mmol/L ascorbic acid, 0.2 mg/mL catalase, 2 mg/mL bovine serum albumin, 50  $\mu$ mol/L [1-<sup>14</sup>C] $\alpha$ -ketoglutarate (specific radioactivity, 55 mCi/mmol), with or without an added prolyl-containing peptide substrate (100  $\mu$ g/mL) (or a lysyl-containing peptide as a control) in 100 mmol/L Tris-chloride buffer, pH 7.8, at 37°C [22]. The reaction was carried out in a microfuge test tube inside a rubber-stoppered 20-mL scintillation vial and started by the addition of enzyme sample and stopped after 40 minutes by adding 100  $\mu$ L of 5% trichloroacetic acid. Tissue solubilizer (500  $\mu$ L, Solvable; Perkin Elmer, Waltham, MA) was added to the scintillation vial outside the microfuge test tube, and the CO<sub>2</sub> evolved was collected for 3 hours. After the microfuge test tube was removed, <sup>14</sup>CO<sub>2</sub> trapped in the solubilizer was estimated by liquid scintillation spectrometry as previously described [18,19]. Activities of  $\alpha$ -ketoglutarate dehydrogenase, glutamate dehydrogenase, and nicotinamide adenine dinucleotide (NAD)– and nicotinamide adenine dinucleotide phosphate (NADP)–dependent isocitrate dehydrogenase were estimated by standard assays as previously described [2,13,23–25].

3. Results

3.1. Insulin release

Table 1 shows that inhibitors of  $\alpha$ -ketoglutarate hydroxylases [26] were potent inhibitors of glucose- and leucine-plus-glutamine–induced insulin release from rat pancreatic islets. Ethyl 3,4 dihydroxybenzoate was the most potent inhibitor followed by 2-methyl  $\alpha$ -ketoglutarate and 3-methyl  $\alpha$ -ketoglutarate.

3.2. Hydroxylase expression

Figs. 1 to 3 show that messenger RNA transcripts for 4 known prolyl hydroxylases and 2 of 3 lysyl hydroxylases, as well as phytanoyl-CoA hydroxylase, are present in human

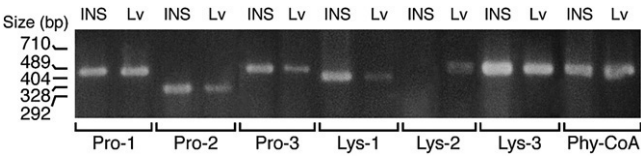


Fig. 1. Reverse transcriptase PCR products of various  $\alpha$ -ketoglutarate hydroxylases in INS-1 cells and liver. Liver is shown as a positive control. The band of the lysine hydroxylase-2 PCR product is a doublet. Lv indicates liver.

and rat pancreatic islets and INS-1 832/13 cells as judged from RT-PCR analysis. Aspartyl hydroxylases were seen in human pancreatic islets, but not in rat pancreatic islets or INS-1 832/13 cells. It is likely that the presence of aspartyl hydroxylase transcripts was due to non-islet cellular contamination of the human islet preparations because aspartyl hydroxylase protein was previously found not to be present in human islets, as judged from immunohistochemistry of human pancreas (J Dinchuk, personal communication). The relative expressions of the hydroxylase genes in  $\beta$ -cells and liver as a positive control are summarized in Table 2.

Immunoblot analysis showed that prolyl hydroxylase proteins P4 H $\alpha$ 1, PHD2, and PHD4 are present in human pancreatic islets and that PHD2 is present in rat pancreatic islets and INS-1 832/13 cells. Prolyl hydroxylases PHD1 and PHD3 were present at very low levels in human islets, and these 2 proteins plus PHD4 could not be detected in rat pancreatic islets (Fig. 4).

3.3.  $\alpha$ -Ketoglutarate hydroxylase activity with endogenous substrates

Table 3 shows that INS-1 cell cytosol has the ability to catalyze decarboxylation of  $\alpha$ -ketoglutarate and that inhibitors of  $\alpha$ -ketoglutarate hydroxylases lower the decarboxylase activity. This indicates that  $\alpha$ -ketoglutarate hydroxylase enzyme activity, as well as endogenous substrate(s) for these hydroxylases, is present in INS-1 cells. It is unlikely that the decarboxylase activity is due to other enzymes that use  $\alpha$ -ketoglutarate, such as  $\alpha$ -ketoglutarate dehydrogenase or glutamate dehydrogenase, because these are mitochondrial enzymes and cytosol was used in these experiments. In

Table 1  
Inhibition of insulin release from rat pancreatic islets by inhibitors of  $\alpha$ -ketoglutarate hydroxylases

Secretagogue	Inhibitor	Insulin release ( $\mu$ U Insulin/5 islets/60 min)
None	None	20 $\pm$ 2
Glucose	None	841 $\pm$ 63
Glucose	1 mmol/L ethyl-3,4-dihydroxybenzoate	48 $\pm$ 5 *
Glucose	10 mmol/L 2-methyl- $\alpha$ -ketoglutarate	54 $\pm$ 7 *
Glucose	5 mmol/L 2-methyl- $\alpha$ -ketoglutarate	291 $\pm$ 21 *
Glucose	10 mmol/L 3-methyl- $\alpha$ -ketoglutarate	315 $\pm$ 29 *
Leucine + glutamine	None	1094 $\pm$ 105
Leucine + glutamine	1 mmol/L ethyl-3,4-dihydroxybenzoate	72 $\pm$ 24 *

Islets were incubated in the presence of the secretagogues for 1 hour. Inhibitors were added 10 minutes before the secretagogues. The concentration of glucose was 16.7 mmol/L, and the concentrations of leucine and glutamine were 10 mmol/L. Results are the mean  $\pm$  SE (n = 6–12 replicate incubations for each condition).

\*  $P < .001$  vs same secretagogue condition without inhibitor.

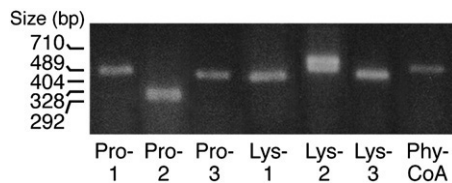


Fig. 2. Reverse transcriptase PCR products of various  $\alpha$ -ketoglutarate hydroxylases in rat pancreatic islets. The band of the lysine hydroxylase-2 PCR product is a doublet.

addition, the inhibitors of the  $\alpha$ -ketoglutarate hydroxylases did not inhibit glutamate dehydrogenase. It is also unlikely that activities of these enzymes would be detected in the enzyme reaction mixture because the endogenous levels of substrates and cofactors for these 2 enzymes would have been diluted >1000-fold in the hydroxylase enzyme reaction mixture. Although  $\alpha$ -ketoglutarate is a substrate for the reverse reaction of NADP-isocitrate dehydrogenase and this enzyme is present in the cytosol of islets and clonal  $\beta$ -cells, this enzyme was not inhibited by high levels of these inhibitors (see below). In addition,  $\alpha$ -ketoglutarate is not decarboxylated in its conversion to isocitrate.

### 3.4. Prolyl hydroxylase activity

To ascertain whether some of the  $\alpha$ -ketoglutarate hydroxylase activity was due to prolyl hydroxylase, INS-1 cell cytosol was fractionated with ammonium sulfate and/or by affinity chromatography on a poly-L-proline column and then tested with inhibitors of  $\alpha$ -ketoglutarate hydroxylases. The fraction precipitated by 30% to 60% ammonium sulfate contained prolyl hydroxylase activity, whereas the zero to 30% fraction and the 60% supernatant fraction contained little or no prolyl hydroxylase activity (Table 4). A supernatant fraction of INS-1 cells was also applied to a poly-L-proline Sepharose-4B column. The column was washed with buffer, and poly-L-proline was added to the column to elute prolyl hydroxylases adhering to the column. Table 5 shows that there was prolyl hydroxylase enzyme activity in the fraction eluted with poly-L-proline ( $M_r$  8900) and that concentrating the eluate increased the enzyme activity in direct proportion to the extent of concentration of the eluate. This fraction contained no activity when (Ile-lys-

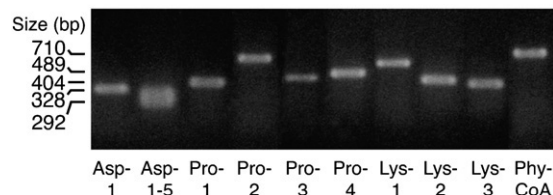


Fig. 3. Reverse transcriptase PCR products of various  $\alpha$ -ketoglutarate hydroxylase genes in human pancreatic islets. The primers used for aspartyl hydroxylase (Asp 1-5) amplify up to 5 different aspartyl hydroxylase transcripts.

Table 2

Relative expression of hydroxylase genes in human and rat pancreatic islets, INS-1 cells, and rat liver

RT-PCR gene product	Relative level of expression by PCR (scale of 0-3)			
	Tissue			
	Human pancreatic islets	Rat pancreatic islets	INS-1	Rat liver
Prolyl hydroxylase 1	2.5	2	3	3
Prolyl hydroxylase 2	1	2.5	2	2
Prolyl hydroxylase 3	1	2.5	2	1
Prolyl hydroxylase 4	1.5	ND	ND	ND
Lysyl hydroxylase 1	2	2	1.5	2
Lysyl hydroxylase 2	1	0	0	0
Lysyl hydroxylase 2 variant	ND	2	1.5	2
Lysyl hydroxylase 3	2.5	2.5	3	2.5
Phytanoyl-CoA hydroxylase	1	2.5	2.5	3
Aspartyl hydroxylase 1	1	ND	0	ND
Aspartyl hydroxylases 1-5	2.5	ND	ND	ND

Expression is summarized from RT-PCR analyses shown in Figs. 1-3 and other experiments. Relative levels are described on a scale of 0 to 3, with 3 representing the highest level and 0 indicating undetectable. ND indicates not done.

gly)<sub>3</sub> was the substrate (data not shown). In addition, adding (pro-pro-gly)<sub>10</sub> (which is a better substrate for prolyl hydroxylase than the longer-chain proline polymer, which was present in the eluate because it was used for elution of the enzyme from the column) to the enzyme reaction mixture increased the prolyl hydroxylase activity of both the unconcentrated and concentrated fractions even more. In contrast, concentrating the flow-through fraction obtained while adding the cytosol sample to the column, or also the

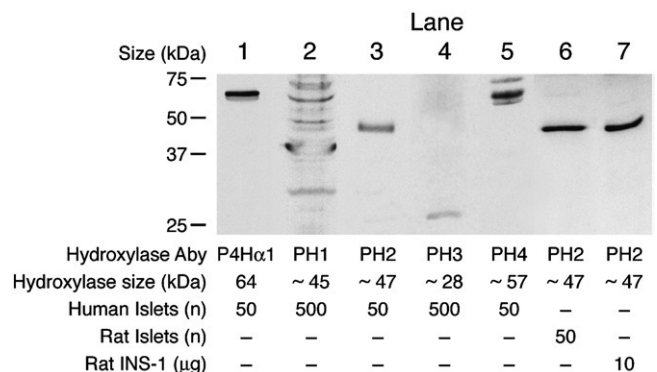


Fig. 4. Immunoblots of prolyl hydroxylases in human and rat pancreatic islets and INS-1 cells. The figure shows lanes from immunoblots probed with antibodies against the various prolyl hydroxylases. The known sizes of the various hydroxylases are shown at the bottom of the figure. To show the absence or a low level of a hydroxylase, 500 islets instead of 50 islets were used. P4H $\alpha$ 1 indicates prolyl hydroxylase P4H $\alpha$ 1; PH 1, PHD prolyl hydroxylase 1; PH 2, PHD prolyl hydroxylase 2; PH 3, PHD prolyl hydroxylase 3; PH 4, PHD prolyl hydroxylase 4.



Table 3

Summary of effects of inhibitors of  $\alpha$ -ketoglutarate hydroxylases on enzyme activity in the presence of endogenous substrates in INS-1 cell cytosol

Incubation condition	Relative enzyme activity (expressed as a % of control = 100%)
Cytosol	100%
No cytosol	10%–18%
Cytosol, heated	5%
Cytosol + 60 mmol/L ZnCl <sub>2</sub>	15%–34%
Cytosol + 5 mmol/L 2-methyl- $\alpha$ -ketoglutarate	25%
Cytosol + 5 mmol/L 3-methyl- $\alpha$ -ketoglutarate	48%
Cytosol + 2.5 mmol/L ethyl-3,4-dihydroxybenzoate	49%

Results are typical of 6 separate experiments.

column wash fraction obtained while washing the column with buffer after adding the sample and before eluting with poly-proline, did not result in an increase in enzyme activity. Furthermore, adding (pro-pro-gly)<sub>3</sub> to either of the latter 2 unconcentrated or concentrated fractions did not increase enzyme activity. Enzyme activity was not present in column fractions collected subsequent to the eluate obtained in the presence of poly-L-proline. Adding ethyl-3,4-dihydroxybenzoate or 2-methyl- $\alpha$ -ketoglutarate to the concentrated polyproline eluate column fraction inhibited the enzyme activity (Table 5) to extents proportional to their effects on insulin release (Table 1).

### 3.5. Little or no inhibition by hydroxylase inhibitors on various enzymes that use $\alpha$ -ketoglutarate

The inhibitors of  $\alpha$ -ketoglutarate hydroxylases were tested for inhibition of other enzymes that use  $\alpha$ -ketoglutarate.  $\alpha$ -Ketoglutarate dehydrogenase was not inhibited by 2-dimethyl- $\alpha$ -ketoglutarate or 3-methyl- $\alpha$ -ketoglutarate at concentrations of up to 10 mmol/L and when assayed at  $\alpha$ -ketoglutarate concentrations of 0.5 or 5 mmol/L. Only when 10 mmol/L ethyl 3,4-dihydroxybenzoic acid (which is 10-fold the highest concentration used in the insulin release study) was added to the enzyme reaction mixture before 5 mmol/L of the substrate  $\alpha$ -ketoglutarate, or when 1 mmol/L ethyl 3,4-dihydroxybenzoic acid was added before 0.5 mmol/L  $\alpha$ -ketoglutarate, was inhibition seen; and then it was only 30%. The 3 inhibitors did not inhibit pure glutamate dehydrogenase from bovine liver or pure NADP-isocitrate dehydrogenase from porcine heart or NAD-isocitrate dehydrogenase in an INS-1 cell mitochondrial supernatant fraction (data not shown).

### 3.6. Lack of effect of inhibitors of hydroxylases on glucose metabolism

2-Methyl- $\alpha$ -ketoglutarate at 1 or 10 mmol/L, which inhibited insulin release up to 67% (Table 1), did not inhibit glucose oxidation. Ethyl-3,4-dihydroxybenzoate (0.25 and 0.5 mmol/L) did not inhibit glucose oxidation by pancreatic islets, and 1 mmol/L ethyl-3,4-dihydroxybenzoate inhibited

glucose oxidation by about 37% (Table 6). Inhibition of insulin release by 1 mmol/L ethyl-3,4-dihydroxybenzoate (94%–97%, Table 1) was proportionately much larger than its inhibition of glucose metabolism.

## 4. Discussion

The RT-PCR analyses of human and rat pancreatic islets and INS-1 832/13 cells indicated that all 3 tissues possess messenger RNA transcripts for prolyl hydroxylases PHD1, 2, and 3 and lysyl hydroxylases 1, 2, and 3, as well as phytanoyl-CoA hydroxylase. The expression of the lysyl hydroxylase 2 was very low in human islets and undetectable in rat islets and INS-1 cells. The RT-PCR is very sensitive and does not accurately estimate the rate of translation of a protein and its level in a tissue. Therefore, the RT-PCR analysis was corroborated by estimating the levels of proteins by immunoblotting. Antibodies for lysyl hydroxylases and phytanoyl-CoA hydroxylase were not available for use in immunoblotting. However, immunoblot analyses of human islets indicated that they possess high levels of proline hydroxylase P4H $\alpha$ 1, PHD2, and PHD4, and low levels of PHD1 and PHD3. Rat islets and INS-1 cells possessed only PHD2 as judged from immunoblot analyses. The presence of prolyl hydroxylase enzyme activity in INS-1 832/13 cells was demonstrated by purifying the enzyme with polyproline affinity chromatography, indicating that the enzyme is present in  $\beta$ -cells.

Inhibitors of  $\alpha$ -ketoglutarate hydroxylases lowered glucose-induced and leucine-plus-glutamine-induced insulin release in rat pancreatic islets and inhibited hydroxylase enzyme activity with endogenous protein substrates in homogenates of INS-1 832/13 cells and also inhibited the affinity-purified INS-1 cell prolyl hydroxylase. Because

Table 4

$\alpha$ -Ketoglutarate hydroxylase activity in various ammonium sulfate fractions of INS-1 cell cytosol

Ammonium sulfate fraction	$\alpha$ -Ketoglutarate hydroxylase activity (pmol [1- <sup>14</sup> C] $\alpha$ -ketoglutarate decarboxylated/mg protein)
Buffer only	0
0%–30%	0.70 $\pm$ 0.06
0%–30% + PGP	0.85 $\pm$ 0.06
30%–60%	4.9 $\pm$ 0.25
30%–60% + PGP	7.3 $\pm$ 0.06
60% Supernatant	0.75 $\pm$ 0.09
60% Supernatant + PGP	0.72 $\pm$ 0.09
Heated 30%–60% + PGP	0.12 $\pm$ 0.01
30%–60% + PGP + 5 mmol/L EDTA	0.84 $\pm$ 0.10

INS-1 cell cytosol was fractionated with ammonium sulfate and dialyzed into 100 mmol/L Tris-Cl buffer, pH 7.8, 20% glycerol, and 1 mmol/L dithiothreitol; and  $\alpha$ -ketoglutarate hydroxylase activity was estimated in the presence and absence of (pro-gly-pro)<sub>10</sub> or EDTA, which chelates iron and inhibits the enzyme. Results are the mean of  $\pm$ SE of triplicate estimates on each fraction. PGP indicates (pro-gly-pro)<sub>10</sub>.

Table 5

Prolyl hydroxylase enzyme activity in various fractions eluted from INS-1 cell cytosol on a polyproline agarose column

Column fraction	$\alpha$ -Ketoglutarate hydroxylase activity		
	(pmol [ $1\text{-}^{14}\text{C}$ ] $\alpha$ -ketoglutarate decarboxylated/25 $\mu\text{L}$ of fraction)		Specific enzyme activity in presence of PGP (nmol [ $1\text{-}^{14}\text{C}$ ] $\alpha$ -ketoglutarate decarboxylated/mg protein)
	Fraction alone	Fraction plus PGP	
Flow through unconcentrated	4.0	3.0	0.05
Flow through concentrated	3.5	3.4	0.01
Wash unconcentrated	4.4	4.2	0.13
Wash concentrated	2.7	1.1	0.01
Polyproline eluate unconcentrated	4.3	14.9	14.9
Polyproline eluate concentrated	18.3	38.6	12.9
Polyproline eluate concentrated plus 1 mmol/L ethyl-3, 4-dihydroxybenzoate	ND	6.1	2.0
Polyproline eluate concentrated plus 10 mmol/L 2-methyl $\alpha$ -ketoglutarate	ND	22.0	7.3

An INS-1 832/13 cell supernatant fraction was applied to a poly-L-proline Sepharose 4B. The column was washed with buffer solution, and prolyl hydroxylase was eluted with poly-L-proline (*M*, 8900) as described under Experimental procedures. The fractions were collected from the column; part of each fraction was concentrated and part of each fraction was assayed for hydroxylase activity in the absence or presence of (pro-gly-pro)<sub>10</sub>. Note that prolyl hydroxylases are known to possess enzyme activity in the presence of long polyproline polymers as substrates, but less than with (pro-gly-pro)<sub>10</sub> as a substrate. The hydroxylation of poly-L-proline, plus hydroxylation of any endogenous proteins, explains enzyme activity in the absence of PGP.

various metabolic enzymes besides  $\alpha$ -ketoglutarate hydroxylase use  $\alpha$ -ketoglutarate as a substrate, the hydroxylase inhibitors were tested for effects on the other enzymes and on glucose oxidation by pancreatic islets. Either the inhibitors did not inhibit the other enzymes and glucose oxidation, or else inhibition of insulin release was proportionately much greater than inhibition of glucose metabolism and the metabolic enzymes. 2-Methyl- $\alpha$ -ketoglutarate and 3-methyl- $\alpha$ -ketoglutarate, which inhibited  $\alpha$ -ketoglutarate hydroxylase activity by about 50% and insulin release by 60% to 90%, did not inhibit  $\alpha$ -ketoglutarate dehydrogenase, NADP- or NAD-dependent isocitrate dehydrogenase enzyme activity, or glucose metabolism. Ethyl-3,4-dihydroxybenzoate inhibited prolyl hydroxylase enzyme activity with endogenous substrates by 50% and insulin release by >90%, but inhibited  $\alpha$ -ketoglutarate dehydrogenase by 30%

Table 6

Little or slight inhibition of glucose oxidation in pancreatic islets by ethyl 3,4-dihydroxybenzoic acid or 2-methyl  $\alpha$ -ketoglutarate

Condition	Glucose oxidation (nmol CO <sub>2</sub> formed/100 islets/90 min)
Glucose alone	3.2 $\pm$ 0.2 (3)
Glucose + 1 mmol/L DKG	3.7 $\pm$ 0.3 (4)
Glucose + 10 mmol/L DKG	3.0 $\pm$ 0.1 (4)
Glucose + 0.25 mmol/L EDB	3.4 $\pm$ 0.2 (3)
Glucose + 0.5 mmol/L EDB	2.9 $\pm$ 0.4 (4)
Glucose + 1 mmol/L EDB	2.0 $\pm$ 0.3 (4)

Islets were incubated with 16.7 mmol/L [ $U\text{-}^{14}\text{C}$ ]glucose (0.4 mCi/mmol) with or without either inhibitor for 90 minutes, and glucose oxidation to CO<sub>2</sub> was measured. Results are the mean  $\pm$  SE, with the number of replicate incubations in parentheses. DKG indicates 2-dimethyl- $\alpha$ -ketoglutarate; EDB, ethyl-3,4-dihydroxybenzoate.

and glucose metabolism by 37%. Although these data support the idea that there are novel actions of  $\alpha$ -ketoglutarate hydroxylases in supporting insulin release in the  $\beta$ -cell, there are reports of inhibition by 3,4-dihydroxybenzoate (not the ethyl ester) of other enzymes, such as hepatic P450 enzymes. However, the inhibition with very high doses was only 20% to 30% [27]. 3,4-Dihydroxybenzoate has also been reported to have a moderate effect on protein kinase C [28] and to have an apoptotic effect in gastric carcinoma cells [29].

Because the known actions of  $\alpha$ -ketoglutarate hydroxylases are in collagen synthesis (P4H $\alpha$ 1) or on activation of gene transcription (HIF prolyl hydroxylases), which are nonacute mechanisms, but the inhibition of insulin release was immediate, the results suggest that there may be acute unknown effects of  $\alpha$ -ketoglutarate hydroxylases in insulin secretion. Insulin secretagogues increase  $\alpha$ -ketoglutarate in pancreatic islets and INS-1 cells [12]. Thus, it is possible that an increase in mitochondrially-generated  $\alpha$ -ketoglutarate derived from insulin secretagogue carbon and translocated to the cytosol may be part of the signal for insulin secretion. We are attempting to identify targets of hydroxylase enzymes in insulin-producing cells. However, this is proving to be difficult because there is no assay for selecting peptides not previously known to undergo hydroxylation.

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