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ARTICLE

# Fuel-induced amplification of insulin secretion in mouse pancreatic islets exposed to a high sulfonylurea concentration: role of the NADPH/NADP$^+$ ratio

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**Abstract**
*Aims/hypothesis* The aim of this study was to examine whether the cytosolic NADPH/NADP$^+$ ratio of beta cells serves as an amplifying signal in fuel-induced insulin secretion and whether such a function is mediated by cytosolic α-ketoglutarate.
*Methods* Pancreatic islets and islet cells were isolated from albino mice by collagenase digestion. Insulin secretion of incubated or perifused islets was measured by ELISA. The NADPH and NADP$^+$ content of incubated islets was determined by enzymatic cycling. The cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) in islets was measured by microfluorimetry and the activity of ATP-sensitive $K^+$ channels in islet cells by patch-clamping.
*Results* Both 30 mmol/l glucose and 10 mmol/l α-ketoisocaproate stimulated insulin secretion and elevated the NADPH/NADP$^+$ ratio of islets preincubated in the absence of fuel. The increase in the NADPH/NADP$^+$ ratio was abolished in the presence of 2.7 μmol/l glipizide (closing all ATP-sensitive $K^+$ channels). However, α-ketoisocaproate, but not glucose, still stimulated insulin secretion. That glipizide did not inhibit α-ketoisocaproate-induced insulin secretion was not the result of elevated $[Ca^{2+}]_c$, as glucose caused a more marked $[Ca^{2+}]_c$ increase. Insulin release triggered by glipizide alone was moderately amplified by dimethyl α-ketoglutarate (which is cleaved to produce cytosolic α-ketoglutarate), but there was no indication of a signal function of cytosolic α-ketoglutarate.
*Conclusions/interpretation* The results strongly suggest that the NADPH/NADP$^+$ ratio in the beta cell cytosol does not serve as an amplifying signal in fuel-induced insulin release. The study supports the view that amplification results from the intramitochondrial production of citrate by citrate synthase and from the associated export of citrate into the cytosol.

**Keywords** Amplification · Glucose · Insulin secretion · α-Ketoisocaproate · α-Ketoglutarate · NADPH · Pancreatic islets · Sulfonylurea

**Abbreviations**

| | |
|---|---|
| BCH | 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid |
| $[Ca^{2+}]_c$ | cytosolic $Ca^{2+}$ concentration |
| $K_{ATP}$ channel | ATP-sensitive $K^+$ channel |
| *t* | time (min) |

## Introduction

Stimulation of insulin secretion by glucose and by some other fuels results from the combination of triggering and amplifying signals produced in the fuel metabolism of pancreatic beta cells [1]. There is strong evidence that an increase in cytosolic ATP and the associated decrease in cytosolic ADP trigger insulin release by closing the ATP-sensitive $K^+$ channels ($K_{ATP}$ channels) in the beta cell plasma membrane [1–3]. The ensuing membrane depolarisation opens voltage-dependent $Ca^{2+}$ channels, thereby raising the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) and

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initiating the exocytosis of insulin [2]. However, about two thirds of glucose-induced insulin secretion are probably due to the amplifying pathway [4]. Two experimental approaches have been employed to demonstrate amplification: all $K_{ATP}$ channels of beta cells are closed by sulfonylurea (e.g. glipizide) [5] or all $K_{ATP}$ channels are held open by diazoxide while the membrane is depolarised by high $K^+$ [6]. The insulin secretion stimulated under these conditions is still enhanced by glucose and other insulin-releasing fuels [1]. Despite considerable efforts, it has remained unclear so far which signals mediate the metabolic amplification. Current signal candidates are an increase in the cytosolic NADPH/$NADP^+$ ratio [7, 8], an increase in cytosolic α-ketoglutarate [9], the export of citrate cycle intermediates to the cytosol [10–13], the accumulation of cytosolic acyl-CoAs [10, 11, 13–16] and an increase in mitochondrial succinyl-CoA [11, 17]. Metabolic amplification does not appear to result from an increase in the cytosolic ATP/ADP ratio [12].

In the beta cell cytosol, two sources yield NADPH [7, 8, 11, 13, 18, 19]. Cytosolic malic enzyme transforms malate + $NADP^+$ into pyruvate + $CO_2$ + NADPH and cytosolic isocitrate dehydrogenase transforms isocitrate + $NADP^+$ into α-ketoglutarate + $CO_2$ + NADPH (Fig. 1). In the cytosol of mouse beta cells, isocitrate is the only important source of NADPH, since the activity of cytosolic malic enzyme is very low [20]. The substrates malate and isocitrate are exported from the mitochondria to the cytosol (Fig. 1) when the activated fuel metabolism provides citrate cycle intermediates [11]. The NADPH/$NADP^+$ ratio in insulin-secreting cells exposed to increasing glucose concentrations rises in parallel with insulin release [7, 8]. However, these findings do not prove a causal link between the changes in NADPH/$NADP^+$ ratio and insulin release. Intracellular application of NADPH via a micropipette was shown to stimulate the exocytosis of insulin granules in whole-cell capacitance measurements [7]. But these findings might not reflect the situation in intact beta cells.

The signal function of citrate cycle intermediates is difficult to verify by extracellular application because these compounds do not cross the beta cell plasma membrane. The membrane-permeable ester dimethyl α-ketoglutarate is converted into α-ketoglutarate in the beta cell (Fig. 1) and elicits insulin release [9, 21]. But it is unclear how α-ketoglutarate is involved in dimethyl α-ketoglutarate-induced insulin secretion and whether its mechanism is compatible with a role as an amplifying signal.

The specific aims of the present study were to examine whether the cytosolic NADPH/$NADP^+$ ratio in beta cells functions as an amplifying signal in fuel-induced insulin secretion and to investigate the role of cytosolic α-ketoglutarate in insulin secretion stimulated by dimethyl α-ketoglutarate.

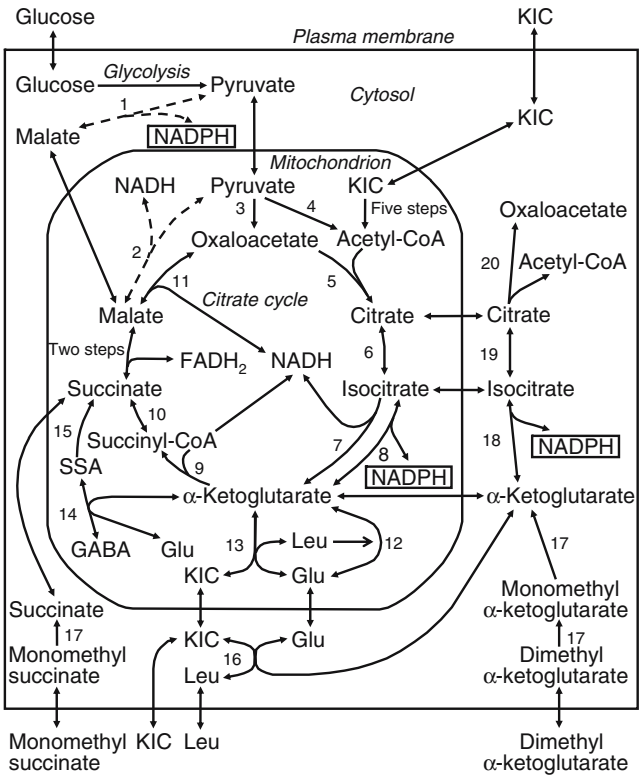


**Fig. 1** Interaction of the citrate cycle with the metabolism of glucose, α-ketoisocaproate, monomethyl succinate and dimethyl α-ketoglutarate in pancreatic beta cells. The plasma membrane and the inner mitochondrial membrane are shown. For clarity, not all compounds participating in the enzyme reactions and transport processes are indicated. The shuttle systems translocating the reducing equivalents of glycolytically formed NADH into the mitochondrion are also not shown. The broken lines indicate that the activities of cytosolic and mitochondrial malic enzyme are very low in mouse beta cells. α-Ketoglutarate is transformed into succinate by sequential reactions catalysed by α-ketoglutarate dehydrogenase and succinyl-CoA synthase, respectively, and perhaps also by GABA transaminase followed by succinate semialdehyde dehydrogenase [50]. α-Ketoisocaproate is transaminated both in the cytosol and the mitochondrion, generating leucine and α-ketoglutarate. Intramitochondrially generated leucine allosterically activates the glutamate dehydrogenase. For further explanations and references, see the text. GABA, γ-aminobutyric acid; Glu, glutamate; KIC, α-ketoisocaproate; Leu, leucine; SSA, succinate semialdehyde. The numbers indicate the following enzymes: *1*, cytosolic malic enzyme; *2*, mitochondrial malic enzyme; *3*, pyruvate carboxylase; *4*, pyruvate dehydrogenase; *5*, citrate synthase; *6*, mitochondrial aconitase; *7*, $NAD^+$-dependent isocitrate dehydrogenase; *8*, $NADP^+$-dependent isocitrate dehydrogenase; *9*, α-ketoglutarate dehydrogenase; *10*, succinyl-CoA synthase; *11*, malate dehydrogenase; *12*, glutamate dehydrogenase; *13*, intramitochondrial aminotransferase; *14*, GABA aminotransferase; *15*, succinate semialdehyde dehydrogenase; *16*, cytosolic aminotransferase; *17*, esterase; *18*, cytosolic isocitrate dehydrogenase; *19*, cytosolic aconitase; *20*, ATP-dependent citrate lyase

## Methods

*Materials and media* Sigma/Fluka (Taufkirchen, Germany) provided α-ketoisocaproate (4-methyl-2-oxopentanoate, sodium salt), α-ketoglutarate (disodium salt dihydrate),

dimethyl α-ketoglutarate (α-ketoglutaric acid dimethyl ester), L-glutamine, glucose 6-phosphate (disodium salt hydrate), 6-phosphogluconate (trisodium salt), 6-phosphogluconate dehydrogenase (from yeast) and imidazole. $NADP^+$ (disodium salt), NADPH (tetrasodium salt), glucose-6-phosphate dehydrogenase (from yeast) and glutamate dehydrogenase (from beef liver) were from Roche (Mannheim, Germany). EDTA and L-cysteine were from Merck (Darmstadt, Germany). Fura-PE3/AM (acetoxymethyl ester), cell culture medium RPMI 1640 and fetal calf serum were supplied by Molecular Probes (Leiden, the Netherlands), Gibco BRL (Gaithersburg, MD, USA) and Biochrom (Berlin, Germany), respectively. All other chemicals were obtained from sources described elsewhere [12, 22, 23]. The media for isolation, perifusion and incubation of pancreatic islets consisted of basal medium (HEPES-buffered Krebs–Ringer bicarbonate medium) containing 2 mg/ml BSA [22]. Other additions to the media are detailed below.

*Isolation and culture of pancreatic islets and islet cells* Albino mice (NMRI) were purchased and bred as described previously [12]. The study was conducted in accordance with the Principles of Laboratory Care. Pancreatic islets from mice of both sexes (9–13 weeks old, fed an unrestricted diet) were isolated by a collagenase digestion technique (in the presence of 5 mmol/l glucose) and hand-picked under a stereomicroscope. Islets were dissociated into single cells by incubation for 10 min in a $Ca^{2+}$-free medium (135 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l $KH_2PO_4$, 1.2 mmol/l $MgSO_4$, 25 mmol/l HEPES, 0.5 mmol/l EGTA, 3 mmol/l glucose, 1% BSA, pH 7.4), subsequent vortex-mixing for 1 min, centrifugation for 1 min at 200 *g* and suspension in culture medium. Isolated islets were cultured in the presence of 5 mmol/l glucose (for microfluorimetric measurements) and islet cells in the presence of 10 mmol/l glucose (for patch-clamp experiments) as described previously [23, 24].

*Measurement of insulin secretion* Insulin secretion from perifused freshly isolated islets was measured as described previously [12]. For static incubations, 50 freshly isolated islets were preincubated in 1 ml of control medium (either in the absence of secretagogue or in the presence of 2.7 μmol/l glipizide) for 60 min at 37°C. Batches of 15 preincubated islets were incubated in 200 μl of control medium or medium plus test compound and the insulin concentration in the media was determined after 40 min at 37°C.

*NADPH and $NADP^+$ measurement* NADPH and $NADP^+$ contents were measured by enzymatic cycling as described by Passonneau and Lowry [25] with minor modifications. Batches of 15 freshly isolated islets were preincubated in 200 μl of control medium (either in the absence of secretagogue or in the presence of 2.7 μmol/l glipizide) for 60 min at 37°C (control period). Then 180 μl of the medium was replaced with control medium or with medium plus test compound and the islets were incubated for 40 min at 37°C. Incubations were stopped by removing 180 μl of medium and placing the incubation tube (0.5 ml polypropylene tube; Sarstedt, Nümbrecht, Germany) in liquid $N_2$. After thawing the tube in an ice bath, 40 μl of ice-chilled NaOH (60 mmol/l+7.5 mmol/l cysteine) was added. The stoppered tube was immediately sonicated as described previously [26] and centrifuged for 1 min at 20,000 *g* (4°C). Aliquots (15 μl) of the supernatant were added to 7.5 μl of NaOH (40 mmol/l+5 mmol/l cysteine; for NADPH determination) and to 7.5 μl of HCl (200 mmol/l; for $NADP^+$ determination). The samples were heated for 10 min at 60°C (to destroy $NADP^+$ and NADPH, respectively) and kept at −80°C (usually for 1–3 days). Addition of 100 μl cycling reagent (100 mmol/l Tris, 80 mmol/l HCl, 7.5 mmol/l α-ketoglutarate, 2 mmol/l glucose 6-phosphate, 0.1 mmol/l ADP, 25 mmol/l ammonium acetate, 0.1% BSA, 6 U/ml glucose-6-phosphate dehydrogenase, 10 U/ml glutamate dehydrogenase, pH 7.4) to each sample started the cycling reaction, which lasted for 1 h at 37°C. The reaction was stopped by heating for 3 min at 97°C and centrifugation for 10 min at 20,000 *g* (4°C). For the fluorimetrical indicator reaction, 90 μl supernatant was mixed with 900 μl indicator reagent [25] without 6-phosphogluconate dehydrogenase. An aliquot (900 μl) of the mixture was transferred to a quartz cuvette in a filter fluorimeter (Eppendorf 1101 M with attachment 1030; Eppendorf, Hamburg, Germany), the fluorescence (excitation at 366 nm, emission at 400–3,000 nm) was followed with a chart recorder and the indicator reaction was started by addition of 6-phosphogluconate dehydrogenase. Recording was continued until the reaction was finished. Appropriate blanks, NADPH standards and $NADP^+$ standards were run in parallel through the entire procedure, including the extraction steps. For each single incubation, the NADPH/$NADP^+$ ratio was calculated from the measured NADPH and $NADP^+$ content.

*Microfluorimetric measurement of the cytosolic $Ca^{2+}$ concentration* Pancreatic islets were cultured on collagen-coated glass cover slips in Petri dishes and were used from day 2 to day 4 after isolation. Fura-PE3/AM was used as $Ca^{2+}$ indicator to minimise dye leakage during the experiments. The loading concentration was 2 μmol/l in basal medium containing 2 mg/ml BSA and 5 mmol/l glucose (45 min at 37°C). As described previously [24], the islets were then perifused at 0.2 ml/min and at 35°C using basal medium (additions as detailed under Results, no BSA) and

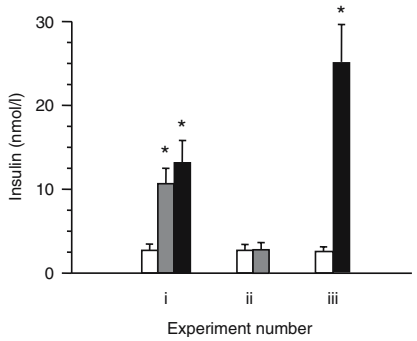


**Fig. 2** Effects of glucose and α-ketoisocaproate on insulin secretion by incubated mouse pancreatic islets. The experimental series consisted of three (*i*) or two (*ii*, *iii*) simultaneous incubations (40 min) in the absence (*i*) or presence (*ii*, *iii*) of 2.7 μmol/l glipizide. The incubation media contained no fuel secretagogue (white bars), 30 mmol/l glucose (grey bars) or 10 mmol/l α-ketoisocaproate (black bars). During the preincubations (60 min in the absence of fuel secretagogue), the media in *ii* and *iii* contained 2.7 μmol/l glipizide. The insulin concentration in the media at the end of the incubation period is indicated. Data are the means±SEM of results from 14 (*i*), 10 (*ii*) or 9 (*iii*) separate experiments. *$p<0.001$ vs corresponding control

the epifluorescence was recorded (six islet subregions evaluated per experiment).

*Patch-clamp experiments* Using single islet cells cultured for 24–72 h, $K_{ATP}$ channel currents in inside-out patches were measured at room temperature (20–22°C) with an experimental design (including calculations) exactly as described previously [23]. No effort was made to determine the proportion of beta cells in our islet cell preparation (intact mouse islets contain >80% beta cells [27]), because the properties of alpha cell $K_{ATP}$ channels are closely similar to those of beta cell $K_{ATP}$ channels [28].

*Statistical analysis* Results are presented as means±SEM. Differences between groups were analysed using the two-tailed *t* test for paired observations. Statistical significance was assumed at $p<0.05$.

## Results

A recent observation led us to conceive the present experiments: in mouse islets exposed to 2.7 μmol/l glipizide (blocking all $K_{ATP}$ channels, [12]), 10 mmol/l α-ketoisocaproate (a fuel secretagogue) amplified insulin secretion, but 30 mmol/l glucose did not, even though both 10 mmol/l α-ketoisocaproate and 30 mmol/l glucose produced similar changes in the islet contents of ATP and ADP [12]. For methodical reasons, the ATP and ADP measurements and the intended NADPH and $NADP^+$ measurements had to be performed with statically incubated islets, whereas insulin secretion was measured in perifused islets. Thus, we had to ascertain that the above observation on secretagogues is also valid for the conditions of our measurements of metabolic intermediates in islets. After preincubating mouse islets for 60 min in the absence of any fuel or secretagogue, 40 min incubations in the presence of 30 mmol/l glucose or 10 mmol/l α-ketoisocaproate stimulated insulin secretion by 3.8- or 4.7-fold, respectively (Fig. 2). A small proportion of the secretory response to α-ketoisocaproate was probably due to direct $K_{ATP}$ channel inhibition [23]. After preincubating mouse islets for 60 min in the absence of fuel but in the presence of 2.7 μmol/l glipizide, 40 min incubations in the presence of 2.7 μmol/l glipizide plus 30 mmol/l glucose did not stimulate insulin secretion (Fig. 2), whereas 40 min incubations in the presence of 2.7 μmol/l glipizide plus 10 mmol/l α-ketoisocaproate stimulated insulin secretion by 9.4-fold (Fig. 2). So the secretory responses of the incubated islets clearly reflect those of the perifused islets (Fig. 2) [12].

**Table 1** Effects of glucose and α-ketoisocaproate on the content of NADPH and $NADP^+$ and the NADPH/$NADP^+$ ratio in mouse islets incubated with or without 2.7 μmol/l glipizide

| Parameter | Without glipizide | | Without glipizide | | With glipizide | | With glipizide | |
|---|---|---|---|---|---|---|---|---|
| | Control | Test | Control | Test | Control | Test | Control | Test |
| Glucose (mmol/l) | 0 | 30 | 0 | 0 | 0 | 30 | 0 | 0 |
| KIC (mmol/l) | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| NADPH (fmol/islet) | 19.9±2.8 | 37.6±4.4[d] | 18.2±2.9 | 31.7±5.9[b] | 21.1±3.6 | 20.5±3.5 | 22.2±5.8 | 21.7±5.0 |
| $NADP^+$ (fmol/islet) | 57.1±6.9 | 58.9±6.0 | 53.6±5.2 | 65.4±4.5 | 45.5±4.7 | 44.6±5.2 | 55.6±6.3 | 57.0±4.6 |
| NADPH/$NADP^+$ ratio | 0.36±0.04 | 0.67±0.09[c] | 0.35±0.05 | 0.49±0.09[a] | 0.46±0.07 | 0.46±0.06 | 0.39±0.08 | 0.37±0.07 |
| *n* | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |

Values are means±SEM for results from separate experiments ($n=8$)

Each of the four experimental series consisted of simultaneous incubations (40 min) in the absence (control) or presence (test) of glucose (30 mmol/l) or α-ketoisocaproate (KIC, 10 mmol/l). During the preincubations (60 min) and incubations, glipizide (2.7 μmol/l) was either absent or present

[a] $p<0.05$, [b] $p<0.02$, [c] $p<0.002$, [d] $p<0.001$ for difference between test and corresponding control

To assess the signal function of the islet NADPH/$NADP^+$ ratio in fuel-induced amplification of insulin secretion, the contents of NADPH and $NADP^+$ were measured in mouse islets. After preincubating islets for 60 min in the absence of any fuel or secretagogue, 40-min incubations in the presence of 30 mmol/l glucose or 10 mmol/l α-ketoisocaproate significantly increased the NADPH/$NADP^+$ ratio in the islets (Table 1). This increase was exclusively due to an increase of the NADPH content, the content of $NADP^+$ remained unchanged. Unexpectedly, the increase in the NADPH content and the NADPH/$NADP^+$ ratio by 30 mmol/l glucose or 10 mmol/l α-ketoisocaproate was abolished when the preincubation and incubation media contained 2.7 μmol/l glipizide (Table 1). Again, there was no fuel-induced change in the $NADP^+$ content and consequently no change in the NADPH/$NADP^+$ ratio. Interestingly, the NADPH content after control incubation, i.e. the continued absence of fuels, was the same in the absence and presence of glipizide. Thus glipizide specifically abolished the fuel-induced increase in NADPH.

To elucidate why mouse islets exposed to 2.7 μmol/l glipizide lost glucose-induced, but not α-ketoisocaproate-induced amplification of insulin secretion [12], $[Ca^{2+}]_c$ was measured as stimulus–secretion coupling signal. Perifusion of mouse islets for 60 min in the absence of nutrients caused a slow increase in $[Ca^{2+}]_c$ which reached a steady state prior to the addition of glucose or α-ketoisocaproate (Fig. 3a). Within 1 min, 30 mmol/l glucose caused a marked transient decrease of $[Ca^{2+}]_c$. After 6 min, the $[Ca^{2+}]_c$ level prior to glucose addition was re-established, then after 15 min a clear further increase was noted. The addition of 10 mmol/l α-ketoisocaproate caused a similarly fast decrease of $[Ca^{2+}]_c$ and again there was an increase after a 6 min time interval. However, the $[Ca^{2+}]_c$ levels remained clearly below the values prior to nutrient addition for the remaining 30 min of perifusion time (Fig. 3a). The addition of 2.7 μmol/l glipizide in the absence of any nutrient caused a strong increase of $[Ca^{2+}]_c$ after a time lag of 2 min (Fig. 3b). The elevated $[Ca^{2+}]_c$ level remained constant until to the addition of the nutrients. Both 30 mmol/l glucose and 10 mmol/l α-ketoisocaproate caused an immediate decrease of $[Ca^{2+}]_c$, the kinetics of which showed a biphasic pattern. Again, the ensuing return to elevated $[Ca^{2+}]_c$ levels was clearly more marked in the presence of glucose than of α-ketoisocaproate (Fig. 3b). As in the previous set of experiments the fura ratio values at the end of the nutrient perifusion were significantly different for glucose and α-ketoisocaproate, whereas no significant difference existed prior to the nutrient exposure.

Dimethyl α-ketoglutarate, which is split into α-ketoglutarate by cytosolic esterases (Fig. 1), stimulated insulin release from pancreatic islets in the absence of other secretagogues [9, 21]. Under our conditions, a moderate amplifying component of the secretory response was observed (Fig. 4). After

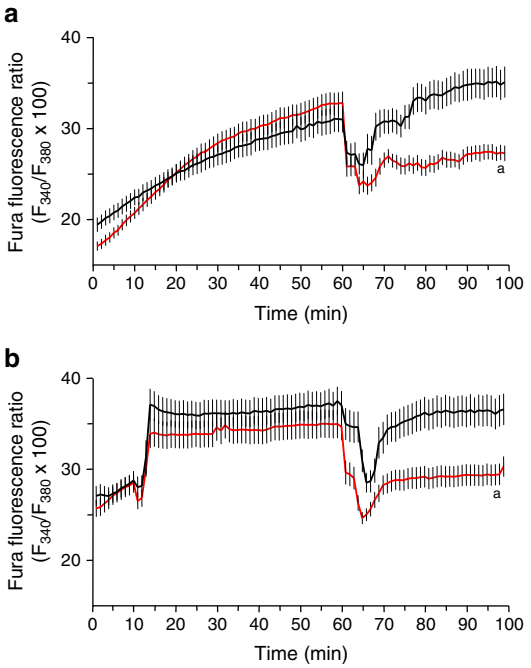


**Fig. 3** Effects of glucose and α-ketoisocaproate on $[Ca^{2+}]_c$ of perifused mouse islets. **a** Fura-loaded islets were perifused with medium containing no glucose for 60 min. Thereafter, either 30 mmol/l glucose (black line) or 10 mmol/l α-ketoisocaproate ([a]red line) was added to the perifusion, which continued for another 40 min. The data are the means±SEM of five (glucose) or six (α-ketoisocaproate) experiments. **b** Fura-loaded islets were perifused with medium containing no glucose for 60 min. From $t$=10 to $t$=100, 2.7 μmol/l glipizide was present. From $t$=60 onwards, either 30 mmol/l glucose (black line) or 10 mmol/l α-ketoisocaproate ([a]red line) was added to the perifusion, which continued for another 40 min. The data are the means±SEM of five (glucose) or six (α-ketoisocaproate) experiments

perifusing islets for 60 min in the presence of 2.7 μmol/l glipizide (control period), perifusion of islets with a medium containing 2.7 μmol/l glipizide plus dimethyl α-ketoglutarate (15 mmol/l) elicited a monophasic increase in insulin release peaking at time ($t$) 62.5 min (Fig. 4). As compared with the secretory rate at $t$=58, the secretory rates were higher from $t$=62.5 to $t$=74 for every experiment. This amplifying effect was weaker than that of 15 mmol/l 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) in conjunction with 2 mmol/l glutamine. The sole application of 2 mmol/l glutamine did not enhance glipizide-induced insulin secretion (Fig. 4), a finding similar to that made previously with glibenclamide (known as glyburide in the USA and Canada) [29].

As stimulation of insulin secretion requires closure of $K_{ATP}$ channels in the beta cell plasma membrane, it is conceivable that α-ketoglutarate derived from dimethyl α-ketoglutarate directly inhibits the $K_{ATP}$ channels. To test this possibility, $K_{ATP}$ channel currents in inside-out patches of islet cell membranes were recorded using the experimental design described previously [23]. The continuous current trace in Fig. 5 revealed pronounced rundown of $K_{ATP}$ channel

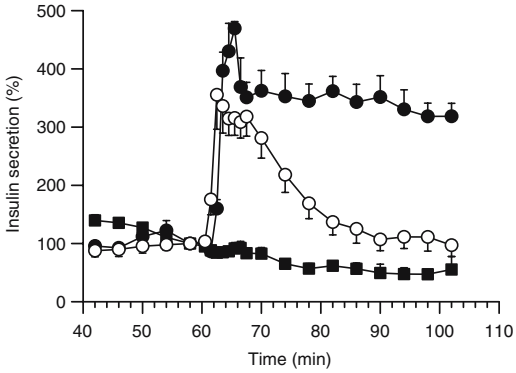


**Fig. 4** Effects of dimethyl α-ketoglutarate, glutamine and BCH on the kinetics of insulin secretion by mouse pancreatic islets. Islets were perifused from $t$=0 to $t$=60 with medium containing 2.7 μmol/l glipizide (control period) and from $t$=61 to $t$=104 (test period) with medium containing 2.7 μmol/l glipizide plus 15 mmol/l dimethyl α-ketoglutarate (white circles) or 2.7 μmol/l glipizide plus 2 mmol/l glutamine (black squares) or 2.7 μmol/l glipizide plus 2 mmol/l glutamine plus 15 mmol/l BCH (black circles). The rate of insulin secretion is expressed as a percentage of the secretion rate at the end of the control period. The data points are the means of six (dimethyl α-ketoglutarate) or three (glutamine, glutamine + BCH) separate experiments (with SEM shown when larger than symbols) and are plotted in the middle of the sampling intervals

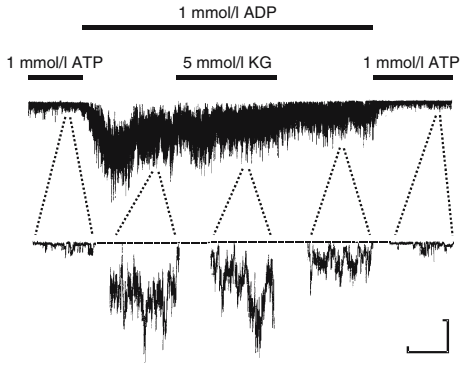


**Fig. 5** Effect of α-ketoglutarate on $K_{ATP}$ channel current in an inside-out patch of a mouse islet cell. Free $Mg^{2+}$ (0.7 mmol/l) was always present in the solutions applied at the cytoplasmic membrane side. Segments of channel activities from the continuous trace are shown below the trace on an expanded time scale. Scale bar (bottom right): horizontal corresponds to 1 min for the continuous trace and to 1 s for the expanded segments; vertical corresponds to 20 pA for the continuous trace and to 10 pA for the expanded segments. Application of intracellular solution containing 1 mmol/l ATP, 1 mmol/l ADP or 1 mmol/l ADP plus 5 mmol/l α-ketoglutarate (KG) was by the bath and at the times/durations indicated (bold horizontal bars). The sodium concentrations in the solutions containing only ATP or ADP were made equal to the sodium concentration in the solution containing α-ketoglutarate by adding NaCl. In this experiment, α-ketoglutarate (5 mmol/l) inhibited the $K_{ATP}$ channel current by only 2.4% of the mean $K_{ATP}$ channel current during the control periods before and after the test period with α-ketoglutarate

activity. Rundown was considered by using the mean current during control periods before and after application of α-ketoglutarate as reference (100%). The $K_{ATP}$ channel current in the presence of 5 mmol/l α-ketoglutarate plus 1 mmol/l ADP was 100.2±4.1% ($n$=6). Thus, α-ketoglutarate did not inhibit the $K_{ATP}$ channels of islet cells.

## Discussion

The findings in this study argue against a role of cytosolic NADPH as an amplifying signal in fuel-induced insulin secretion. The data leading to this conclusion were obtained by use of a high (2.7 μmol/l) glipizide concentration as a tool blocking all beta cell $K_{ATP}$ channels. It has previously been shown in mouse islets perifused with 2.7 μmol/l glipizide (no fuel during the pre-stimulatory period) that 10 mmol/l α-ketoisocaproate amplified insulin secretion, whereas 30 mmol/l glucose did not [12]. A similar difference was also observed, when insulin secretion was determined in islets incubated under conditions corresponding to those during our measurements of metabolic intermediates (Fig. 2). The present results support the view that this difference was not due to a lower $[Ca^{2+}]_c$ in the presence of 30 mmol/l glucose (Fig. 3).

In the absence of fuels, whole-islet and mitochondrial NADPH/$NADP^+$ ratios were similar in islets and isolated islet cells, respectively [30, 31]. Both ratios rose in parallel in the presence of glucose. Glucose also raised the cytosolic NADPH/$NADP^+$ ratio [32, 33]. These findings allow conclusions to be drawn from the whole-islet NADPH/$NADP^+$ ratio about the mitochondrial and the cytosolic NADPH/$NADP^+$ ratios. The prevention by glipizide of glucose- and α-ketoisocaproate-induced elevation of the islet NADPH/$NADP^+$ ratio (Table 1) argues against a signal function of the cytosolic NADPH/$NADP^+$ ratio in fuel-induced amplification of insulin secretion. This is concluded because glipizide failed to suppress α-ketoisocaproate-induced insulin release (Fig. 2) [12]. It is very unlikely that the resistance to glipizide resulted from an as yet undetected direct amplifying effect of α-ketoisocaproate as such, since inhibition of α-keto acid transamination abolished amplification by α-ketoisocaproate [23]. Moreover, amplification of glipizide-induced insulin secretion was also seen with BCH, which is not structurally related to α-ketoisocaproate [12]. The prevention by glipizide of an increase in the islet NADPH/$NADP^+$ ratio also argues against formation of isocitrate by reversal of the mitochondrial $NADP^+$-dependent isocitrate dehydrogenase reaction (Fig. 1) [12], since reversal of this reaction requires NADPH levels to be elevated by the mitochondrial electrochemical proton gradient [34].

The glucose- and α-ketoisocaproate-induced increase in the islet NADPH/$NADP^+$ ratio resulted from an elevation of the NADPH content, whereas the $NADP^+$ content remained unchanged (Table 1). These observations are assumed to reflect a dual action of the fuels. First, the fuels stimulate NAD regeneration from precursors and conver-

sion of some of the NAD into NADP [35]. These reactions require ATP. Second, a more reduced state of the NADPH–$NADP^+$ couple is brought about. The expected decrease of the $NADP^+$ content seems to be hidden by the synthesis of $NADP^+$.

In isolated pancreatic islets and insulin-secreting cells, glucose and α-ketoisocaproate were consistently found to raise the NADPH/$NADP^+$ ratio (Table 1) [7, 8, 13, 19, 30, 36, 37]. How could the presence of 2.7 μmol/l glipizide abolish this effect? The reason is probably the depolarisation of the inner mitochondrial membrane potential by the high glipizide concentration. The $K_{ATP}$ channels in the beta cell plasma membrane are half-maximally inhibited by 7 μmol/l tolbutamide or 4 nmol/l glipizide (the therapeutic free plasma concentrations are 6–25 μmol/l and 4–40 nmol/l, respectively; 6 or 2% of the total plasma concentration of tolbutamide or glipizide, respectively, are free) [22]. At much higher concentrations, sulfonylureas uncouple oxidative phosphorylation [38–40]. In mouse beta cells perifused in the absence of exogenous fuels, 500 μmol/l tolbutamide generated a small depolarisation of the inner mitochondrial membrane potential [41]. The depolarisation amounted to only 10% of the depolarisation induced by the mitochondrial protonophoretic uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μmol/l) and seemed to be associated with a decrease in the cytosolic ATP concentration. The depolarisation was believed to result either from direct inhibition of the mitochondrial $K_{ATP}$ channels or from uncoupling of oxidative phosphorylation. In mouse islets incubated in the absence of exogenous fuels, tolbutamide (about 400 μmol/l free concentration) slightly stimulated oxygen consumption [26, 42]. Decrease in ATP content induced by high sulfonylurea concentrations was observed in mouse and rat islets incubated in the absence of exogenous fuels or in the presence of low glucose concentrations [43–46]. Mild mitochondrial depolarisation is the likely reason why glipizide caused glucose and α-ketoisocaproate to induce only a small increase in the islet ATP/ADP ratio [12]. The contrasting effects of glipizide on the secretory responses to glucose on the one hand and to α-ketoisocaproate on the other hand (Fig. 2) [12] probably indicate that reactions involved in the generation of the amplification signal by glucose need a higher ATP/ADP ratio than reactions following formation of the signal [47]. Another likely consequence of a low ATP/ADP ratio is an inhibition of ATP-dependent regeneration of NADP (see above). In conjunction with this effect, mitochondrial depolarisation and the associated increase in consumption of reducing equivalents (NADH and NADPH) in the respiratory chain can explain why glipizide prevented the fuel-induced rise in the islet content of NADPH.

Evidence has been presented that α-ketoisocaproate, some related α-keto acid anions and the nonmetabolisable leucine analogue BCH amplify insulin secretion by enhancing the intramitochondrial formation of α-ketoglutarate and thereby stimulating the citrate cycle in beta cells [12, 23, 48]. The present study supports the view that formation of citrate from α-ketoglutarate via reversal of the $NADP^+$-dependent isocitrate dehydrogenase reaction does not take place in the presence of 2.7 μmol/l glipizide (see above). Therefore, citrate production induced by α-ketoglutarate represents citrate synthesis from oxaloacetate and acetyl-CoA by the citrate synthase (Fig. 1). Acetyl-CoA comes from degradation of the fuel secretagogue (e.g. α-ketoisocaproate, Fig. 1) or from endogenous sources (e.g. endogenous fatty acids in the case of BCH) [49]. It has been proposed that succinyl-CoA generated from succinate or α-ketoglutarate (Fig. 1) is important for insulin secretion due to its reaction with mitochondrial acetoacetate to form succinate and acetoacetyl-CoA, a precursor of acetyl-CoA (reaction not shown in Fig. 1) [17]. However, it is unclear whether this reaction is significant for BCH-induced amplification of insulin secretion, since low acetoacetate levels are expected in the absence of exogenous fuels or presence of glutamine (Fig. 4) [12].

Not only NADPH, but also α-ketoglutarate is provided by isocitrate exported to the cytosol (Fig. 1). Direct stimulation of insulin secretion by α-ketoglutarate has been proposed to explain the insulin-releasing effect of dimethyl α-ketoglutarate [9]. This membrane-permeable ester is split via monomethyl α-ketoglutarate (two isomers) into α-ketoglutarate (Fig. 1) [21]. But as initiation of insulin release requires closure of $K_{ATP}$ channels in the beta cell plasma membrane, the lack of direct $K_{ATP}$ channel inhibition by α-ketoglutarate (Fig. 5) suggests channel inhibition by activation of mitochondrial energy metabolism and the associated changes in the cytosolic ATP and ADP concentrations. The amplification of insulin secretion by dimethyl α-ketoglutarate cannot result from mitochondrial uptake of α-ketoglutarate, which occurs in exchange for malate and causes no net increase in citrate cycle intermediates of mouse beta cells, as suggested by findings for monomethyl succinate (Fig. 1) [12, 19, 20]. It is unlikely that glutamate formed by transamination of cytosolic α-ketoglutarate mediates the amplifying effect of dimethyl α-ketoglutarate. The islet content of glutamate, but not the amplification of insulin secretion, is much more enhanced by 2 mmol/l glutamine than by 10–20 mmol/l dimethyl α-ketoglutarate (Fig. 4) [21, 29]. Whatever is the precise mechanism of insulin release by dimethyl α-ketoglutarate, our present observations do not support a signal function of cytosolic α-ketoglutarate.

α-Ketoglutarate-induced citrate production causes a net increase in citrate cycle intermediates and enhanced export of these intermediates from the beta cell mitochondria into the cytosol [11]. In the cytosol, citrate, but not the other intermediates, apparently mediates amplification of fuel-

induced insulin secretion. First, the cytosolic NADPH/$NADP^+$ ratio and the cytosolic α-ketoglutarate level probably do not serve as amplification signals (this study). Therefore mitochondrial export of isocitrate and α-ketoglutarate does not appear to amplify insulin release. Second, the very weak amplification of insulin secretion by monomethyl succinate in mouse islets [12] argues against a signal function of cytosolic succinate, fumarate and malate (Fig. 1). Fumarate is formed from succinate and malate and is exported to the cytosol in exchange for succinate (not shown in Fig. 1). Third, succinyl-CoA and oxaloacetate cannot cross the inner mitochondrial membrane. Cytosolic citrate is converted into acetyl-CoA and oxaloacetate by the ATP-dependent citrate lyase (Fig. 1). It has been proposed that compounds enhancing insulin secretion exist among acyl-CoAs and lipids, which are synthesised from acetyl-CoA, using NADPH [10, 11, 14–16]. The lack of a signal function of the cytosolic NADPH/$NADP^+$ ratio does not mean that the supply of NADPH is insufficient for these biosynthetic reactions.

In conclusion, the present study strongly suggests that the NADPH/$NADP^+$ ratio in the beta cell cytosol does not serve as an amplifying signal in fuel-induced insulin release. The findings support the view that amplification results from intramitochondrial production of citrate by the citrate synthase. Citrate exported to the cytosol probably mediates an increase in insulin secretion by promoting the generation of acyl-CoAs and lipids, some of which are putative amplifying signals.

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