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Oxo-4-methylpentanoic acid directs the metabolism of GABA into the citric acid cycle in rat pancreatic islets

Short title: oxo-4-methylpentanoic acid promotes the oxidative metabolism of GABA in islets

Key words: oxo-4-methylpentanoic acid, GABA (γ -aminobutyric acid), α -amino acids, islets, glucose, L-leucine

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¹ Abbreviations used: GABA: γ -aminobutyric acid; OMP: Oxo-4-methylpentanoic acid; SSA: semialdehyde succinic acid; GABA-T: GABA transaminase; GDH: glutamate dehydrogenase.

SYNOPSIS

Oxo-4-methylpentanoic acid (OMP) stimulates by itself a biphasic secretion of insulin whereas L-leucine requires the presence of L-glutamine. L-glutamine is predominantly converted to GABA in rat islets and L-leucine seems to promote its metabolism in the “GABA shunt” (Fernández-Pascual S. et al, *Biochem. J.* 379, 721-729, 2004). We have investigated how 10 mM OMP affects L-glutamine metabolism to uncover possible differences with L-leucine that might help to elucidate whether they share a common mechanism of stimulation of insulin secretion. At variance with L-leucine, OMP alone stimulated a biphasic insulin secretion in rat perfused islets and decreased the islet content of GABA without modifying its extracellular release irrespective of the medium L-glutamine concentration. It was transaminated to L-leucine whose intracellular concentration did not change because it was very efficiently transported out of the islet cells. L-[U-¹⁴C]Glutamine, 0.5 and 10.0 mM, conversion to ¹⁴CO₂ was enhanced by 10 mM OMP within 30 and 70 %, respectively. Gabaculine (250 μM), a GABA transaminase inhibitor, suppressed OMP-induced oxygen consumption but not L-leucine or glucose stimulated respiration. It also suppressed OMP-induced decrease of islet GABA content and OMP-induced increase in insulin release. These results support the view that OMP promotes islet metabolism in the “GABA shunt” generating 2-oxoglutarate, in the branched-chain α-amino acid transaminase reaction, which would in turn trigger GABA deamination by GABA transaminase. OMP, but not L-leucine, suppressed islet semialdehyde succinic acid reductase activity and this might shift the metabolic flux of the “GABA shunt” from γ-hydroxybutyrate to succinic acid production.

INTRODUCTION

L-Leucine is unique, amongst all the other natural α-amino acids, in its ability to trigger a transient stimulation of insulin secretion [1] that becomes sustained in the presence of L-glutamine [2]. The common explanation is that L-leucine activates allosterically β-cell glutamate dehydrogenase (GDH) [3,4] and, in that way, favours L-glutamate

oxidative metabolism in the citric acid cycle [5]. It is then understandable that secretion is only sustained in the presence of L-glutamine which provides the substrate (L-glutamate) for GDH. By contrast, the deaminated derivative of L-leucine, oxo-4-methylpentanoic acid (OMP), is capable of triggering a biphasic secretion of insulin in islets in the complete absence of L-glutamine [6,7]. It has been argued that OMP might be transaminated in β -cells to L-leucine which would activate GDH and make more substrate (2-oxoglutarate) available for the Krebs-cycle [8]. However, this does not explain the strikingly different potency, and L-glutamine-dependence, between the amino acid and the α -keto acid as insulin secretagogues. A different secretagogue behaviour of L-leucine and OMP has also been reported in islets cultured at high glucose [9] or in islets from diabetes-susceptible mice (10). Therefore, it does not seem unreasonable to propose that OMP might stimulate insulin secretion by a different mechanism than L-leucine, not relying exclusively in its transamination to the α -amino acid.

We have supplied evidence [4] indicating that L-leucine might facilitate the metabolism of L-glutamine-derived L-glutamate in the Krebs cycle and the "GABA-shunt": L-Glutamine-derived L-glutamate is predominantly metabolized to GABA by glutamate acid decarboxylase (GAD) in islets, this probably being the reason for it not acting as a secretagogue, and L-leucine is capable of decreasing the islet content of GABA elevated by glutamine. GABA may accumulate in synaptic-like microvesicles from which it may be released by exocytosis [11]. Alternatively, GABA might be transaminated to semialdehyde succinic acid (SSA) by GABA transaminase (GABA-T) [12] which would then be either oxidized to succinic acid by the SSA-dehydrogenase [13] or reduced to γ -hydroxybutyrate by a SSA-reductase [14]. This work was aimed at investigating the capacity of oxo-4-methylpentanoic acid to modify the metabolism of glutamine (glutamate), main GABA precursor(s), in isolated rat islets. For this purpose, the effect of oxo-4-methylpentanoic acid on islet glutamine oxidation, islet amino acid profile (islet content of GABA, L-glutamate, and L-aspartate), and the different enzyme activities of the "GABA-shunt" (GAD, GABA-T, SSA-dehydrogenase and SSA-reductase) were explored.

EXPERIMENTAL

Materials

Collagenase P was obtained from Roche Diagnostics S.L. (Barcelona, Spain). Bovine serum albumin (BSA) and most of the organic compounds were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain). Rat insulin was from Linco Research, Inc. (St. Charles, Missouri, U.S.A.). Na^{[125]I} was obtained from Amersham Iberica S.A. (Madrid, Spain); D-[U-¹⁴C]glutamine and NaH¹⁴CO₃ were from New England Nuclear (Itisa Biomedica S.A., Madrid, Spain) or American Radiolabeled Chemicals (Itisa Biomedica S.A., Madrid, Spain). Inorganic compounds and organic solvents were obtained from Merck Farma y Química S.A. (Madrid, Spain).

Methods

Insulin secretion

Islets were isolated from the pancreas of male Wistar-albino rats (250 g BW) by collagenase digestion. Insulin secretion was studied in perfused or incubated islets. Two groups, each of 40 rat islets, were perfused in parallel and at a flow rate of 0.5 ml/min with Krebs-Ringer, buffered with 0.5 mM NaHCO₃ and 20 mM HEPES, supplemented with 0.5 % BSA (KRBH), and heated at 37⁰C. The perfusion protocol was similar in all the experiments. After a pre-perfusion period of 45 minutes under basal conditions (in the absence of nutrients), the perfusion medium was switched to one containing the test substances and maintained for the next 30 minutes. Finally, the medium was changed back to pre-perfusion conditions where it was maintained for the last 25 minutes. The perfusate was collected at 1 minute intervals during the last 60-70 minutes of perfusion and its insulin concentration radioimmunologically measured. Alternatively, three batches each of ten islets were incubated in 1 ml of KRBH at 37⁰C for 120 minutes and the concentration of insulin released in the incubation medium was radioimmunologically measured. Pig insulin was radio-iodinated with Na¹²⁵I [19] and rat insulin was used as a standard in the radioimmunoassay of insulin. Insulin antiserum was kindly provided by Dr. Janove Sehlin from the Department of Medical Cell Biology at the University of Umeå (Sweden).

L-Glutamine oxidation

Oxidation of L-glutamine was measured as the production of ¹⁴CO₂ from L-[U-¹⁴C]glutamine as previously described [15] and corrected according to the recovery of NaH¹⁴CO₃ which was measured three times during each experiment.

Amino acid measurements

Islet α -amino acids were separated by reverse-phase HPLC after precolumn derivatization with o-phthalaldehyde [16] and quantified by fluorescence detection. Islets (groups of 20) were incubated for 60 minutes at 37⁰C in 100 μ l of the same buffer as that used in perfusions. Incubations were stopped by placing the tubes containing the islets on ice. The medium was aspirated and the islets were washed twice with 100 μ l of cold PBS. Finally, 100 μ l of 35 % (w/v) 5-sulfosalicylic acid were added in order to extract islet amino acids. Final extracts were kept at -80⁰C until their amino acid content was determined. Twelve amino acids were regularly identified and separated in the extracts and they were measured with a sensitivity close to 1 pmol (Asp, Glu, Ser, Gln, His, Gly, Thr, Arg, Tau, Ala, Tyr, and GABA). Occasionally, the gradient elution profile of the chromatographic separation was changed in order to separate and quantify also L-leucine. The amount of protein was measured in the extracts with the method of Lowry [17] and BSA was used as the standard. In experiments aimed at investigating the release of amino acids, islets (groups of 30) were incubated for 60 minutes at 37⁰C in 70 μ l of the same buffer as that used in perfusions. After stopping the incubation on ice, 50 μ l of the supernatant was withdrawn and stored frozen until amino acid measurement was performed. The remaining medium was discarded and the islets were processed as described before for the measurement of their amino acid content.

Enzyme activities

SSA-dehydrogenase and SSA-reductase activities were measured as the production of NADH or the consumption of NADPH, respectively, in homogenates of rat islets or cerebellum, at 0.2 mM SSA. Tissue samples (islets or cerebellum) were homogenized in a homogenization buffer (50 mM NaHPO₄ and 2 mM EDTA, pH= 7.2) containing: leupeptin (31 μ g/ml), aprotinin (4 μ g/ml), PMSF (40 μ l of a 50 mM solution in DMSO /ml), β -mercaptoethanol (0.7 μ l/ml), and Triton-X100 (0.5 %, vol/vol). Isolated islets were homogenized by sonication (four times, ten strokes at 10-20 % of the cycle and the minimum potency of a Branson sonifier 450); for extraction of cerebellum samples, a higher potency (5) and frequency (50 %) of sonication were used. The homogenates were centrifuged at 40,000 rpm for 20 minutes and the supernatants stored at + 4⁰C until the measurement was performed. Enzyme activities were measured in the same buffer used for homogenization but supplemented with β -mercaptoethanol (5 mM), oxamate

(lactate dehydrogenase inhibitor, 2 mM), rotenone (5 μ M), Triton-X100 (0.5 %, vol/vol), BSA (5 mg/ml), and either NADPH (50 μ M) or NAD⁺ (1 mM). The total volume was 500 μ l and the reaction was initiated by addition of 5 μ l of 20 mM SSA, after mixing 470 μ l of the buffer plus 25 μ l of islet homogenate (approximately 6 islets/ μ l) or a convenient dilution of the cerebellum homogenate (10 μ l, at 250 mg/ml) in the cuvette. Absorbance at 340 nm evolved linearly during several minutes and its slope was calculated and taken as a measure of enzyme activity.

GABA transaminase activity of islet homogenates was measured as the formation of GABA from saturating concentrations (20 mM) of L-glutamate and succinic acid semialdehyde and as the formation of L-glutamate from saturating concentrations of GABA (20 mM) and 2-oxoglutarate (10 mM) during 15 minutes of incubation at 37⁰C. The enzyme activity was extracted in the same homogenization buffer described above for the assay of SSA-related activities, supplemented with leupeptin (25 μ g/ml), aprotinin (25 μ g/ml), and PMSF (20 μ l/ml of a 50 mM solution in DMSO). Isolated islets were sonicated (approximately 1 islet/ μ l) under the conditions described above for the other islet enzyme activities. The enzyme activity was measured in the same buffer as described above for homogenization but supplemented with the adequate substrates. The reaction was stopped by the addition of 5-sulfosalicylic acid (3.5 % w/v, final concentration). The amounts of GABA or L-glutamate formed were measured by fluorescence detection after precolumn derivatization with o-phthalaldehyde and HPLC separation.

Oxygen tension measurements

Intra-islet oxygen tension was measured as previously described [18]. Briefly, individual islets were attached to a poly-lysine coated coverslip, subsequently used as a floor in a Sykes-Moore perfusion chamber [19] thermostated at 37⁰C. The perfusion medium was supplemented with 1 mg/ml albumin and contained (in mM): NaCl 125, KCl 5.9, MgCl₂ 1.2, CaCl₂ 1.28, and HEPES 25, titrated to pH 7.4 with NaOH and thermostated at 37⁰C. Oxygen tension was measured by a modified Clark-type microelectrode, with its tip positioned inside the islets. A stereomicroscope was used to control the penetration depth of the electrode to approximately 50 μ m. After an equilibration period of 10 minutes, islets were perfused in the absence or presence of 250 μ M gabaculine for 30 minutes. Subsequently, either 10 mM OMP, 20 mM glucose

or 10 mM L-leucine were introduced into the perfusion buffer in the continued absence or presence of 250 μ M gabaculine and oxygen tension was monitored for an additional 20 minutes.

Statistics

All the experimental data are here presented as the mean values \pm SE, and the numbers of separate experiments are given in parentheses. Statistical comparisons were performed with non-paired, two-tailed Student's t tests.

RESULTS

Insulin secretion

The secretagogue capacity of oxo-4-methylpentanoic acid (OMP) was explored in a dynamic system of perfused rat islets as well as in batch type incubations. Figure 1 shows that 10 mM OMP stimulates a biphasic secretion of insulin by itself. L-Glutamine (0.5 or 10 mM) slightly enhanced the height of the first phase but did not modify at all the second phase. However, no significant differences between the overall effects induced by each of the two L-glutamine concentrations compared with the control could be demonstrated. In islets pre-perfused for 45 minutes with 250 μ M gabaculine, the subsequent secretory response to 10 mM OMP in the continuous presence of the drug was significantly reduced (26 %, $p < 0.008$) as compared with control islets (Figure 2). The amount of insulin released during the first phase of secretion (first ten minutes of stimulation) was not modified although the height of its peak (minute 20th) was significantly reduced (2.1 ± 0.2 , $n=16$, vs. 2.7 ± 0.1 ng/ min x 40 islets, $n=17$; $p < 0.007$). The overall amount of insulin secreted during the second phase (last twenty minutes of stimulation) was decreased by 33% (22.1 ± 2.6 , $n=14$, vs. 33.0 ± 1.9 ng/ 40 islets, $n=17$; $p < 0.002$) and its rate showed a trend to diminish progressively with time. In fact, in islets incubated with 10 mM OMP for 2 hours, 250 μ M gabaculine induced a greater decrease (-47%) of the secretory response (9.5 ± 1.2 , $n=6$, vs. 17.9 ± 2.2 pg / min x islet, $n=6$; $p < 0.008$).

Islet amino acid profile

As reported earlier (4), changes of the extracellular L-glutamine concentration in the range 0.1 to 10.0 mM induce variations of the intracellular concentration of L-glutamine and other amino acids deriving from the amine, like L-glutamate, L-aspartate, and GABA. In order to compare their respective turnover rates and to investigate the effect of OMP, the evolution of islet amino acids contents (Asp, Glu, and GABA) was followed for three hours. The results (Figure 3) show that the turnover rate of islet GABA, calculated as the slope of the linear decrease of the amino acid with time, is higher than that of L-aspartate (4.6 vs. 2.6 pmol / μ g protein x hour) and L-glutamate (not modified at all). L-Aspartate transamination to L-glutamate might compensate its decarboxylation to GABA and contribute to keep islet L-glutamate constant. OMP (10 mM) strongly decreased islet GABA content (-40%) and accelerated islet GABA turnover (Figure 3), calculated as the mean of the differences in amino acid content between control and OMP-stimulated islets during three hours, by 77% (8.1 vs. 4.6 pmol/ μ g protein x hour). Furthermore, OMP accelerated L-aspartate by 65% (4.4 vs. 2.6 pmol/ μ g protein x hour), and L-glutamate by 400% (2.5 vs. 0.5 pmol/ μ g protein x hour). OMP-induced acceleration of islet GABA turnover might be attributed either to its increased vesicular accumulation and exocytosis or to its accelerated metabolism in the "GABA-shunt".

In order to distinguish between these two alternatives and to check whether GABA release is dependent on its intracellular content, islets were pre-loaded for 1 hour with different L-glutamine concentrations (0, 0.5, and 10.0 mM) and then incubated with, or without (control islets), 10 mM OMP for a second hour in the absence of L-glutamine. The latter, at millimolar concentrations, interfered with the chromatographic separation of amino acids in the incubation medium. Figure 4A reveals that the magnitude of GABA release from control islets depends on the pre-loading L-glutamine concentration in the incubation medium which also conditions a concentration-related increase of the islet GABA content (Fig. 4C). A similar pattern of variation was also observed for L-aspartate and L-glutamate (Fig 4A and 4C). However, L-aspartate and GABA showed the greatest relative increases of their islet content after pre-loading at 10 mM L-glutamine (4- and more than 5-fold, respectively, versus 1.5 for Glu) whereas the relative elevation of their release rate (4- and 3-fold, respectively) was much lower than that of Glu and Gln (6.6- and 19.4-fold, respectively). However, islet L-glutamine content did not change significantly with the extracellular L-glutamine concentration

maintained during the pre-loading phase. Taurine, one of the most abundant but metabolically inert amino acid in islets, did not show any significant variations of its islet content or release at the different L-glutamine concentrations, in contrast to GABA, L-glutamate or L-aspartate (results not shown).

Addition of 10 mM OMP modified substantially the islet content and release of amino acids after the different pre-loading L-glutamine concentrations (Fig. 4B and 4D). The most striking modification of islet content was a close to 50% suppression of GABA levels (Fig 6D) at every L-glutamine concentration whereas L-aspartate and L-glutamate contents were either slightly decreased or not modified at all, respectively. Neither the islet content of L-glutamine, nor that of L-leucine (data not shown), varied with the extracellular concentration of the amine during preloading conditions. Even though 10 mM OMP did not modify the tissue concentration of L-leucine, it induced a strong release of the amino acid whose magnitude was dependent on the L-glutamine concentration to which islets were pre-exposed (Fig 4B). By contrast, L-leucine release did not vary with the L-glutamine concentration and it was at least 6-fold lower (Fig. 4A) in control islets. Like in those islets not exposed to OMP during the second hour of incubation, the release of L-glutamine also augmented with the concentration of the amine used during preloading. Similarly, the release of GABA increased with the concentration of L-glutamine at which islets were preloaded and it was not substantially modified by 10 mM OMP: it only decreased GABA release after preloading with 10 mM L-glutamine (23.6 ± 2.1 , $n=7$, vs. 31.8 ± 2.0 pmol/ μg protein, $n=8$; $p < 0.02$) (Fig. 4A and 4B). However, 10 mM OMP decreased consistently the release of L-aspartate within almost 2-fold irrespective of the L-glutamine concentration used during islet preloading.

As gabaculine considerably decreased OMP -stimulated insulin secretion (see Figure 2), it was checked whether the inhibition of GABA transaminase with 250 μM gabaculine interfered with KIC-induced modification of islet GABA content. 10 mM OMP decreased by more than 40 % the islet content of GABA and within only 20 % that of L-aspartate but the content of L-glutamate remained constant (Figure 5). 250 $\mu\text{mol/l}$ Gabaculine completely blocked OMP-induced decrease of islet GABA but did not affect the content of other amino acids (Figure 5). Gabaculine (250 μM) alone increased significantly the islet content of GABA (37.2 ± 5.2 , $n=6$, vs. 23.8 ± 2.7 pmol/ μg protein,

n=6; p< 0.04). These results are compatible with the known specificity of gabaculine as a GABA-transaminase inhibitor and suggest that OMP -induced decrease of islet GABA content might be due to its increased metabolism in the “GABA shunt”.

In order to strengthen the evidence that OMP-induced decrease of islet GABA content is not the result of its increased exocytotic release, the effect of 250 $\mu\text{mol/l}$ diazoxide was investigated: the drug did not modify the level of GABA (9.1 ± 1.4 , n=6, vs. 9.4 ± 1.1 pmol/ μg x protein, n=6, N.S.) or any other α -amino acid (results not shown) in the presence of OMP.

L-Glutamine oxidation

$^{14}\text{CO}_2$ -Production from L-[U- ^{14}C]glutamine was previously shown to have an ED_{50} value close to 0.5 mM, to reach an apparent maximum rate at 10 mM, and to be stoichiometric with the amount of GABA synthesised (4). The oxidation rate of 0.5 mM L-glutamine was shown to be diminished by 10 mM L-leucine which was, however, unable to modify the oxidation of the amine at 10 mM (4). By contrast, it is shown in Figure 6 that 10 mM OMP increased significantly the rate of $^{14}\text{CO}_2$ -production from 0.5 and 10 mM L-[U- ^{14}C]glutamine by 27 and 66%, respectively. Allylglycine (10 mM), a precursor of a competitive inhibitor of glutamate acid decarboxylase (GAD), decreased the rate of glutamine oxidation at 0.5 mM (by more than 30 %) but had no effect at 10 mM L-glutamine (Figure 6). In contrast, 250 μM gabaculine, a known GABA transaminase inhibitor added simultaneously with the other substrates, did not modify the rate of glutamine oxidation in the presence of 10 mM OMP (Figure 6). Succinic acid dimethyl ester (SAD, 10 mM), a membrane permeable form of the dicarboxylic acid (21), reduced, probably by isotopic dilution, the oxidation rate of 0.5 mM L-glutamine alone (4.6 ± 0.2 , n=6, vs. 6.2 ± 0.4 pmol / islet x hour; p<0.005) and in the presence of 10 mM OMP (6.3 ± 0.3 , n=6, vs. 7.9 ± 0.2 pmol / islet x hour, n=6; p<0.002).

Islet enzyme activities of the “GABA shunt”

The effect of OMP on the enzyme activities of the “GABA shunt” was investigated in search of the hypothetical mechanism responsible of an increased islet metabolism of GABA that might result in an increased substrate inflow (succinic acid) into the Krebs cycle. Since it is already known that OMP does not modify islet GAD activity (4) we

investigated the effect of OMP on GABA transaminase activity (GABA-T). It was measured in islet homogenates in either the amination and deamination directions. The maximum rate of GABA deamination was 2 to 3-fold higher than the amination rate. OMP (10 mM) reduced GABA-T activity. The reduction in amination (-34%) being greater than that of deamination (-17%) (Table 1). Gabaculine (40 μ M), a known GABA-T inhibitor, reduced very strongly the rate of GABA amination (-90%) and proportionately less the deamination rate (-69%).

The products of GABA-T activity are L-glutamate and SSA. SSA may be further oxidized in the "GABA shunt" by a dehydrogenase that uses NAD^+ as cofactor and generates succinic acid plus NADH, both susceptible of being further oxidised in the Krebs cycle and the respiratory chain. Alternatively, SSA may be reduced by a specific reductase that uses NADPH as a cofactor and produces γ -hydroxybutyrate and NADP^+ . Both enzyme activities (SSA-dehydrogenase and SSA-reductase) were measured in islet homogenates and cerebellum, the latter being a tissue rich in GABAergic neurons (Table 1). The specific enzyme activities were much higher in islets than in cerebellum: SSA-dehydrogenase activity was almost 70-fold and SSA-reductase about 10-fold greater. Another important difference between the two tissues was that the SSA-dehydrogenase/SSA-reductase activity ratio was close to 1 in islets and around 6 in cerebellum. The rates of both enzyme activities were several hundred fold greater than the measured GABA-T activity in islets. SSA-reductase activity was strongly reduced by 10 mM OMP in either islet (-76%) and cerebellum (-47%) homogenates whereas SSA-dehydrogenase activity was not affected at all (Table 1). None of the two enzyme activities was modified by 10 mM L-leucine in either cerebellum and islet homogenates (results not shown).

Islet respiration

Oxygen consumption gives information on the overall rate of tissue metabolism. It was measured in rat islets to confirm whether OMP was actually increasing the rate of metabolism in the "GABA shunt" investigating the effect of a GABA-T inhibitor, 250 μ M gabaculine, on both islet respiration and insulin release. The temporal kinetics of evolution of islet oxygen tension differs considerably according to the substrate supporting respiration: Oxygen tension falls abruptly when islets respire in the presence

of either L-leucine and OMP whereas it diminishes more slowly in time when high glucose is the respiration substrate (Figure 7). After exposing islets for 30 minutes to gabaculine, 10 mM OMP-induced decrease of the oxygen tension was partially (-59%, $p < 0.016$) suppressed (Figure 7A) whereas the stimulation of respiration by either 20 mM glucose or 10 mM L-leucine were not modified (Figures 7B and 7C, respectively).

DISCUSSION

It is confirmed in this work that OMP stimulates by itself a biphasic secretion of insulin in rat perfused islets [6,7] and that the presence of L-glutamine does not induce any overall potentiation of the secretory response. This means that, at variance with L-leucine ([2,4], OMP is not dependent on extracellular L-glutamine to trigger a biphasic insulin secretion. It has been previously reported that OMP greatly increases the amount of L-leucine accumulating in incubated islets but no distinction was made between medium and islets [22]. Our results confirm that OMP increases L-leucine production in islets but they also show that the amino acid does not accumulate intracellularly and is very efficiently transported outward down its concentration gradient. At variance with other amino acids (GABA, L-aspartate and L-glutamate) which do not seem to permeate islet cells [4], 10 mM extracellular L-leucine increased several fold its islet content that remained, however, 5-fold lower than the levels of GABA, L-aspartate, L-glutamate and L-glutamine obtained at 10 mM extracellular L-glutamine [4]. These data support the view that L-leucine is transported through the plasma membrane of islet cells by a passive, mediated process. Failure to demonstrate any intracellular accumulation of L-leucine argues against the possibility that the allosteric activation of glutamate dehydrogenase is the main mechanism underlying OMP stimulation of insulin secretion. However, it cannot be completely discarded that a L-leucine gradient was developed in any subcellular compartment (mitochondria), not detected in overall cell measurements, that might increase the α -amino acid concentration to values compatible with a stimulation of glutamate dehydrogenase [23]. Similarly to L-leucine, L-glutamine seemed to be passively transported out of the islet cells down its concentration gradient and this process efficiently prevented its intracellular accumulation. Given that islet L-glutamine uptake may also limit its rate of metabolism [4], it seems plausible that it is transported through the plasma membrane by a passive mediated mechanism with predominance over an active, Na^+ -dependent system.

Islet GABA content exhibited the highest turnover rate among the amino acids that are synthesised from L-glutamine (Asp, Glu) and it is accelerated by OMP. The turnover rate of GABA is determined by the balance among its rate of synthesis and the sum of its exocytotic release and its metabolism. As OMP did not induce any substantial modification of GABA release but strongly decreased (close to 50%) its islet content, we conclude that the 4-oxo acid improves “GABA shunt” metabolism. Even though the release of GABA was not affected by OMP, it was positively related to its islet content itself depending on the extracellular L-glutamine concentration. This might simply reflect that a greater availability of the γ -amino acid results in its increased accumulation in the synaptic like microvesicles of the β -cells. These data reinforce previously reported evidence indicating that GABA release from β -cells is positively correlated with its content [24].

10 mM OMP increased $^{14}\text{CO}_2$ -production from 0.5 and 10.0 mM L-[U- ^{14}C]glutamine by almost 30 and 70 %, respectively. This increment might reflect an increased decarboxylation of L-glutamine-derived L-glutamate to GABA or an increased oxidation in the Krebs cycle. As OMP did not increase islet GAD activity and it strongly diminished islet GABA content, one is tempted to speculate that it favours the metabolism of GABA at any enzymatic step in the “GABA shunt”. This is strengthened by the observation that 0.5 mM L-glutamine oxidation is significantly reduced by allylglycine, a precursor of a competitive inhibitor of GAD. The 2-oxoglutarate generated in the transamination of OMP to L-leucine, or in the proper metabolism of the 4-oxo acid [7,25], might be used to trigger the transamination of GABA to SSA in the reaction catalyzed by GABA-T. An increased concentration of islet 2-oxoglutarate above a certain level has been demonstrated to lower the GABA content [4]. Alternatively, 2-oxoglutarate might be directly decarboxylated to succinyl-CoA in the citric acid cycle. The predominance of one of these two alternatives (metabolism in the “GABA shunt” versus the citric acid cycle) will depend on the ratio of the 2-oxoglutarate K_m -values for the two implicated enzymes (GABA-T and 2-oxoglutarate dehydrogenase) and on the tissue ratio of enzymes’ activities. Moreover, if OMP acted as a competitive inhibitor of the 2-oxoglutarate dehydrogenase complex in islets as reported in rat brain (26), the 2-oxo acid would favour “GABA-shunt” metabolism over the direct oxidation of 2-oxoglutarate in the citric acid cycle.

Neither GABA-T, nor SSA-dehydrogenase activities were activated by OMP which, on the other hand, very strongly diminished SSA-reductase activity in both islets and cerebellum. However, L-leucine failed to modify either SSA-reductase or SSA-dehydrogenase activity in islets and cerebellum homogenates. In islets, SSA-reductase activity, responsible of SSA conversion into γ -hydroxybutyrate, is of similar magnitude than SSA-dehydrogenase, the enzyme activity catalyzing the transformation of SSA into succinic acid. Therefore, OMP, at variance with L-leucine, might favour succinic acid production in the “GABA shunt” and ultimately facilitate the oxidation of GABA carbons in the Krebs cycle. In support of this conclusion, pre-incubation with gabaculine, a GABA-T inhibitor, partially suppressed the increased consumption of oxygen by islets induced by OMP whereas it had no effect on either glucose or L-leucine stimulated respiration. The inhibition of OMP-supported respiration (-59%) correlated with a 33 % (perifused islets) or 47% (incubated islets) decrease of the insulin secretory response to the 4-oxo acid. The greater suppression of respiration than secretion might be explained either by the existence of non-metabolic factors partially responsible of the secretory response to OMP or to unspecific metabolic effects of gabaculine exerted on both β - and non- β cells in the islets. Moreover, this partial and concomitant suppression of islet respiration and secretion by a relatively high extracellular concentration of gabaculine (250 μ M) might reflect the drug failure to reach effective intracellular concentrations that completely antagonize GABA-T activity and hence islet GABA usage. Alternatively, other metabolic pathways, like the oxidation of the 4-oxo acid itself and of the 2-oxoglutarate generated during its transamination to L-leucine, might also be contributing to the stimulation of secretion. The balance between these two alternatives would be definitively settled if one could measure with a sufficiently sensitive method the effect of gabaculine on an end product of the “GABA-shunt” like γ -hydroxybutyric acid.

It is generally accepted that an increased glutamate oxidative metabolism in the citric acid cycle secondary to glutamate dehydrogenase activation by L-leucine predominantly contributes to the biphasic stimulation of insulin secretion in the presence of L-glutamine [3,4]. This mechanism might explain the hyperinsulinism observed in children with gain of function mutations of the glutamate dehydrogenase gene [27,28]. OMP, at variance with L-leucine, is capable of stimulating a biphasic secretion of insulin in the absence of L-glutamine. Moreover, it is efficiently transaminated to L-

leucine, without apparently increasing the overall islet content of the α -amino acid. However, similarly to L-leucine but by a different mechanism (branched-chain α -amino acid transamination versus GDH activation), it seems to favour islet metabolism in the “GABA shunt” by increasing the availability of 2-oxoglutarate. Finally, in contrast to L-leucine, OMP might shift the metabolic flux of the “GABA shunt” from γ -hydroxybutyrate to succinic acid production by suppression of SSA-reductase activity. Succinic acid metabolism in the citric acid cycle might in turn elevate cellular ATP levels and activate the mechanism of insulin secretion in the β -cell.

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LEGENDS TO FIGURES

Figure 1. Effects of 10 mM oxo-4-methylpentanoic acid (OMP) alone and in combination with L-glutamine, on insulin secretion by rat perfused islets.

Groups of 40 islets each, pre-perfused without substrates for 45 minutes, were stimulated for 30 minutes (between vertical broken lines) with 10 mM OMP alone (triangles, n=6) or together with 0.5 mM (circles, n= 15) or 10 mM (squares, n= 6) L-glutamine. Pre-perfusion conditions were then re-established during the last 25 minutes. Symbols represent mean values \pm SE and the number of observations (n) is given above, at the right of each symbol.

Figure 2. Effect of 250 μ M gabaculine, a GABA transaminase inhibitor, on the insulin response induced by 10 mM oxo-4-methylpentanoic acid (OMP) in perfused rat islets.

Groups of 40 islets each, pre-perfused without substrates (control, open triangles, n=17) or with 250 μ M gabaculine (filled circles, n=14) for 45 minutes, were stimulated for 30 minutes (between vertical broken lines) with 10 mM OMP alone (open triangles, control) or together with 250 μ M gabaculine (filled circles). Pre-perfusion conditions were then re-established during the last 25 minutes. Symbols represent mean values \pm SE and the number of observations (n) is given above, at the right of each symbol.

Figure 3. Effect of 10 mM oxo-4-methylpentanoic acid (OMP) on the time evolution of the islet amino acid content in the absence of L-glutamine.

Three groups, each of 30 islets, were incubated at 37 $^{\circ}$ C without any substrate (circles) or OMP (triangles) for different times (1,2 or 3 hours). At the end of incubation, the medium was sucked off and the islets were washed twice with saline and extracted with 35 % (w/v) 5-sulfosalicylic acid. Twelve amino acids were separated and quantified by HPLC after their derivatization with o-phthalaldehyde. Only the changes of the

amino acids known to be synthesized from L-glutamine and showing more significant variations were represented (A, Asp; B, Glu; C, GABA). The symbols correspond to mean values \pm SE of 6 different experiments.

Figure 4. Effect of 10 mM oxo-4-methylpentanoic acid (OMP) on islet amino acid content and release after pre-incubation with L-glutamine (Gln).

Three groups, each of 30 islets, were pre-incubated for 1 hour at 37⁰C with different Gln concentrations (0, 0.5, or 10.0 mM, as indicated numerically in the horizontal axis of each graph or represented as white, grey, or black columns, respectively). After discarding the medium and washing the islets twice with saline, they were further incubated for a second hour in the absence of substrates (A and C) or in the presence of 10 mM OMP (B and D). At the end of incubation, the medium (A and B) and islets (C and D) were separated for the measurement of their respective content of amino acids (open squares, GABA; filled squares, Asp; open circles, Glu; filled circles, Gln). The symbols and columns represent mean values \pm SE of 6-8 different experiments. Each value was statistically compared with its corresponding control obtained in the absence of Gln during the pre-incubation phase (§ $p < 0.05$, * $p < 0.02$, † $p < 0.01$, ‡ $p < 0.001$).

Figure 5. Effect of oxo-4-methylpentanoic acid (OMP) and gabaculine on islet amino acid content in the absence of L-glutamine.

Three groups, each of 20 islets, were incubated without any substrate (white columns, control) or with 10 mM OMP alone (grey columns) or combined with 250 μ M gabaculine (black columns) at 37⁰C for 60 minutes. At the end of incubation, the medium was sucked off and the islets were washed twice with saline and extracted with 35 % (w/v) 5-sulfosalicylic acid. Twelve amino acids were separated and quantified by HPLC after their derivatization with o-phthalaldehyde. Only the changes of those amino acids known to be synthesized from L-glutamine are represented. The columns correspond to mean values \pm SE of 6 experiments.

Figure 6. Effect of 10 mM oxo-4-methylpentanoic acid (OMP) and 250 μ M gabaculine on islet L-glutamine oxidation.

Three groups, each of 20 islets, were incubated at 37°C for 60 minutes with either 0.5 (white columns, n=6) and 10.0 mM (black columns, n=4) L-[U-¹⁴C]glutamine alone or together with OMP or GCUL. L-Glutamine oxidation was measured as the production of ¹⁴CO₂. The columns represent mean values ± SE of a number of experiments that is given above within parenthesis. (*p<0.02, †p< 0.01, and ‡ p<0.001, compared with the corresponding control, in the presence of L-glutamine alone).

Figure 7. Islet oxygen tension of rat islets perfused in the presence oxo-4-methylpentanoic acid (OMP), glucose or L-leucine alone or in combination with gabaculine.

Individual islets were perfused in the absence (squares) or presence (triangles) of 250 μM gabaculine for 30 minutes (last 10 minutes shown in panels). At 10 minutes, either 10 mM OMP (A), 20 mM glucose (B) or 10 mM L-leucine (C) was added to perfusion medium containing or not gabaculine. Symbols represent means ± SE for 6 independent experiments in each group.

Table 1. Effect of oxo-4-methylpentanoic acid (OMP) and gabaculline (GCUL) on semialdehyde succinic acid (SSA) reductase and dehydrogenase activities and GABA-transaminase (GABA-T) activity of rat islet and cerebellum homogenates.

SSA-dehydrogenase and SSA-reductase activities of islets and cerebellum homogenates were measured as the production of NADH or the consumption of NADPH, respectively, in homogenates of rat islets or cerebellum, at 0.2 mM SSA. GABA-T activity of islet homogenates was measured as the formation of GABA from saturating concentrations of L-glutamate and succinic acid semialdehyde (20 mM) and as the formation of L-glutamate from saturating concentrations of GABA (20 mM) and 2-oxoglutarate (10 mM). (N.S., non significant)

SSA REDUCTASE activity (nmol NADPH/min per mg prot)						
	ISLETS			CEREBELLUM		
	mean ± SEM	n	p	mean ± SEM	n	p
CONTROL	4312,0 ± 190,3	6	--	62,0 ± 6,5	6	--
10 mM OMP	1046,0 ± 118,8	6	<0,001	33,2 ± 5,1	6	<0,01

SSA DEHYDROGENASE activity (nmol NADH/min per mg prot)						
	ISLETS			CEREBELLUM		
	mean ± SEM	n	p	mean ± SEM	n	p
CONTROL	3754,7 ± 419,5	6	--	366,6 ± 38,3	6	--
10 mM OMP	4137,2 ± 285,0	5	N.S.	359,7 ± 40,8	6	N.S.

GABA-T activity (pmol/15 min per islet)						
	DEAMINATION VELOCITY			AMINATION VELOCITY		
	mean ± SEM	n	p	mean ± SEM	n	p
CONTROL	50,7 ± 1,9	6	--	19,7 ± 1,1	14	--
10 mM OMP	42,1 ± 1,7	6	<0,01	13,1 ± 1,0	6	<0,005
40 µM GCUL	15,5 ± 1,5	6	<0,001	2,1 ± 0,3	5	<0,001

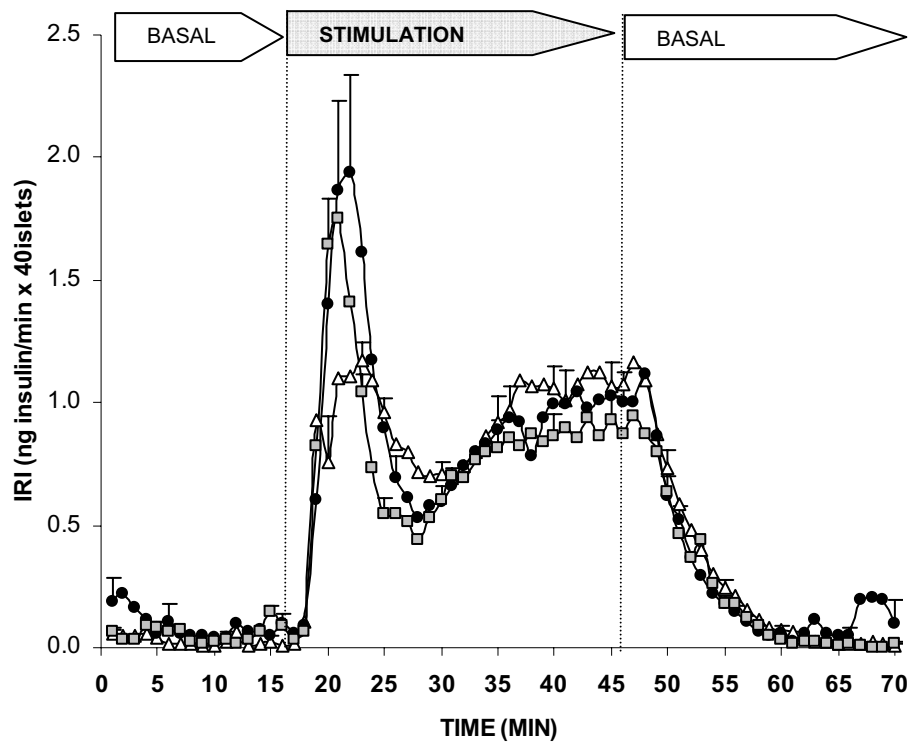


Fig 1

1

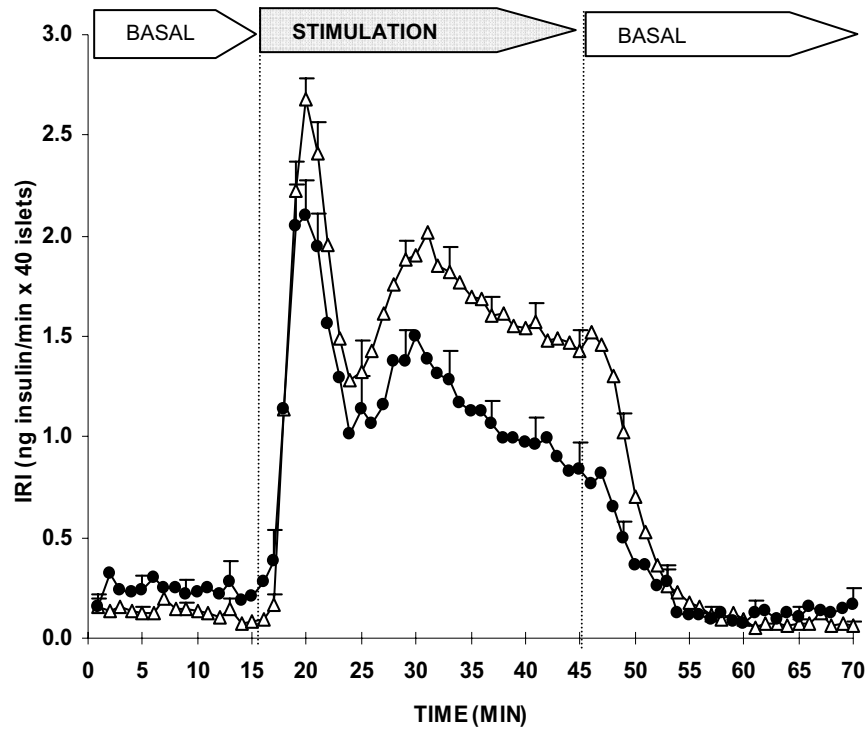


Fig 2

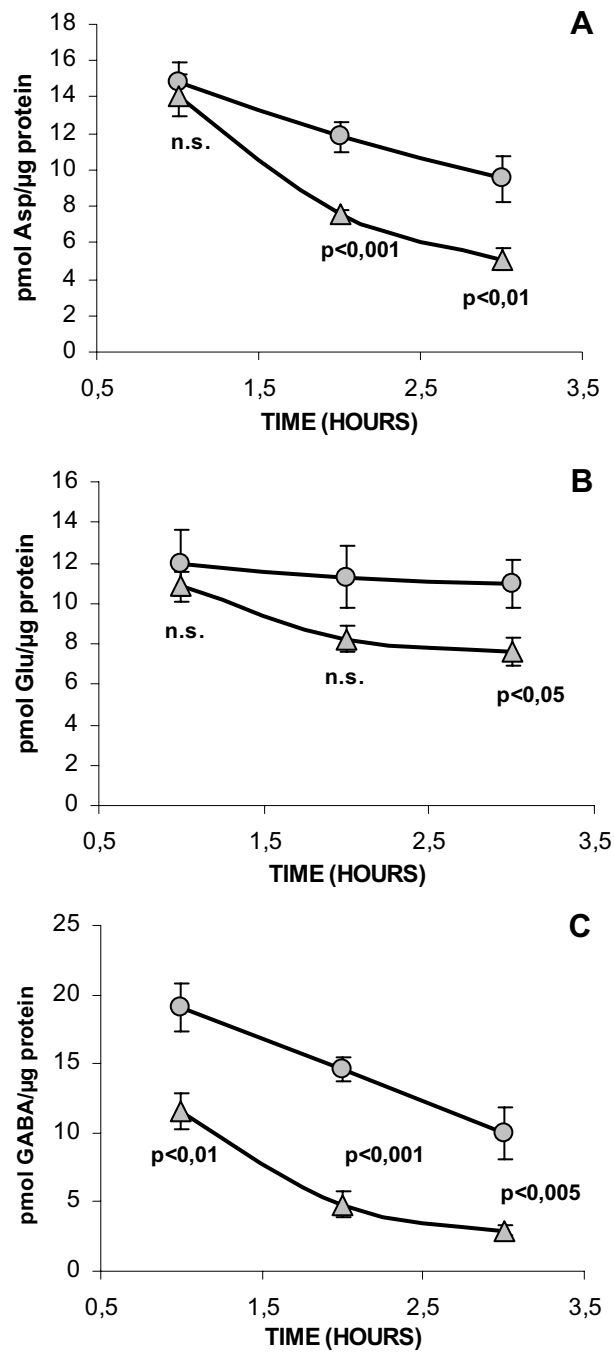


Fig. 3

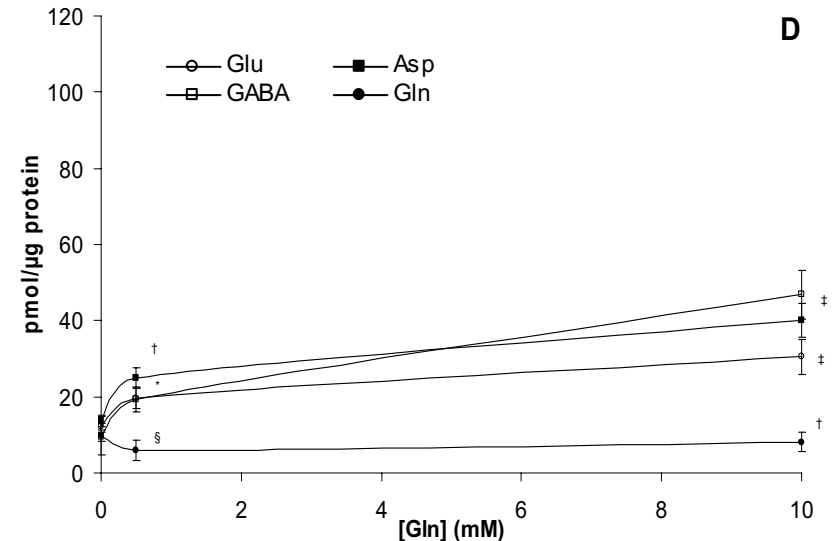
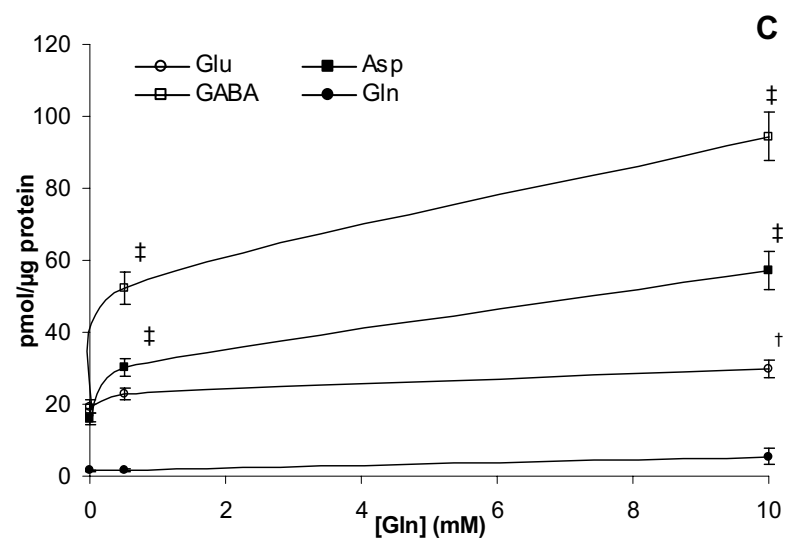
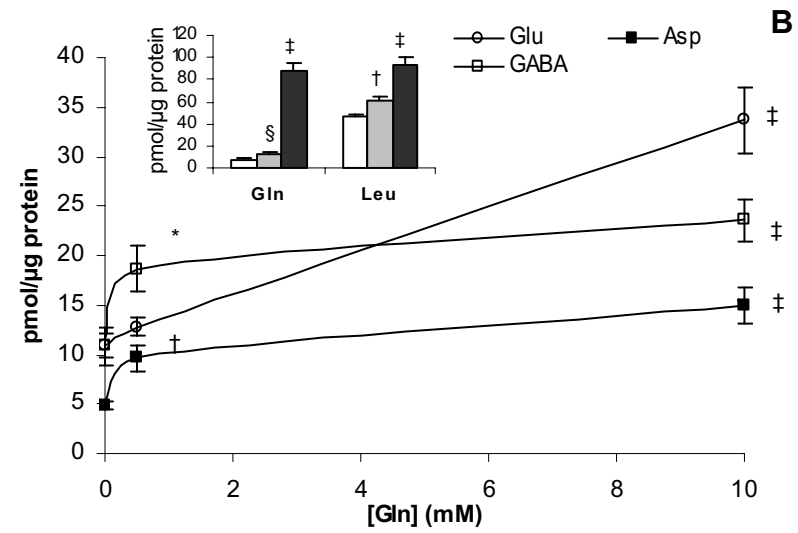
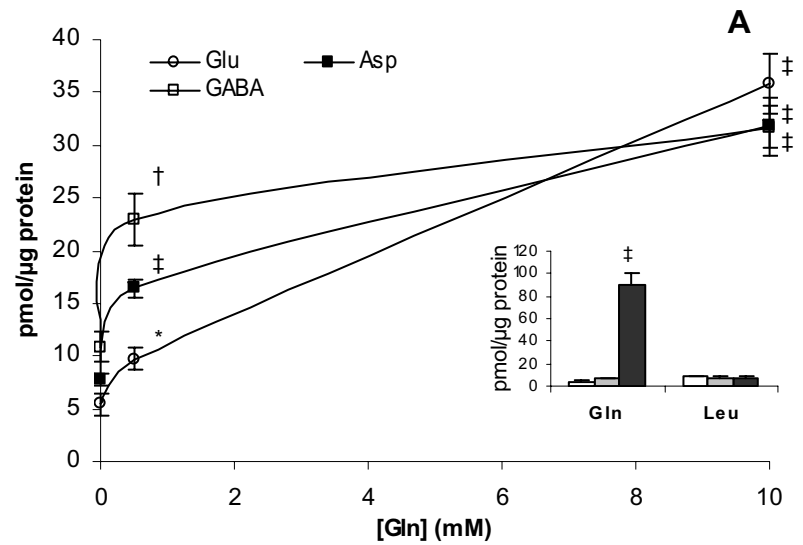


Fig. 4

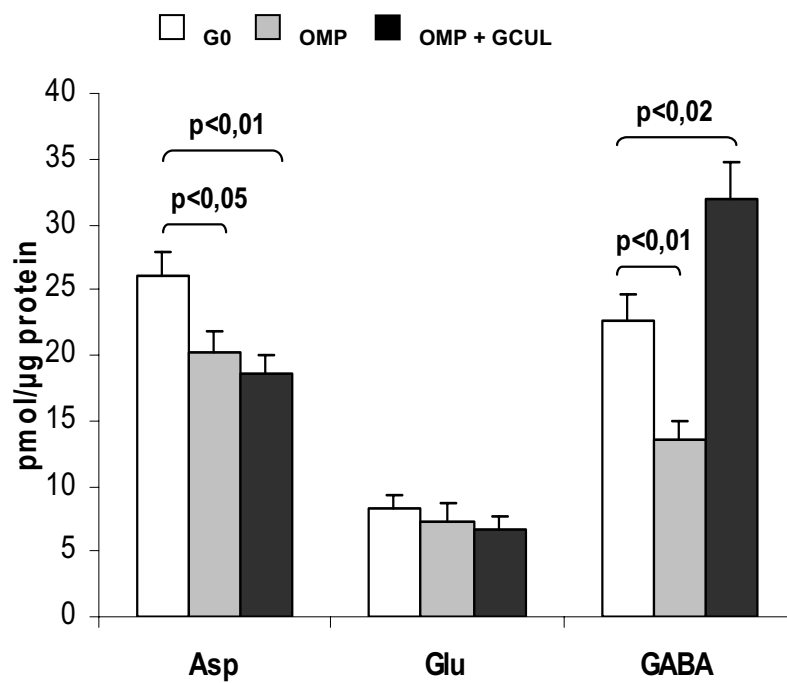


Fig. 5

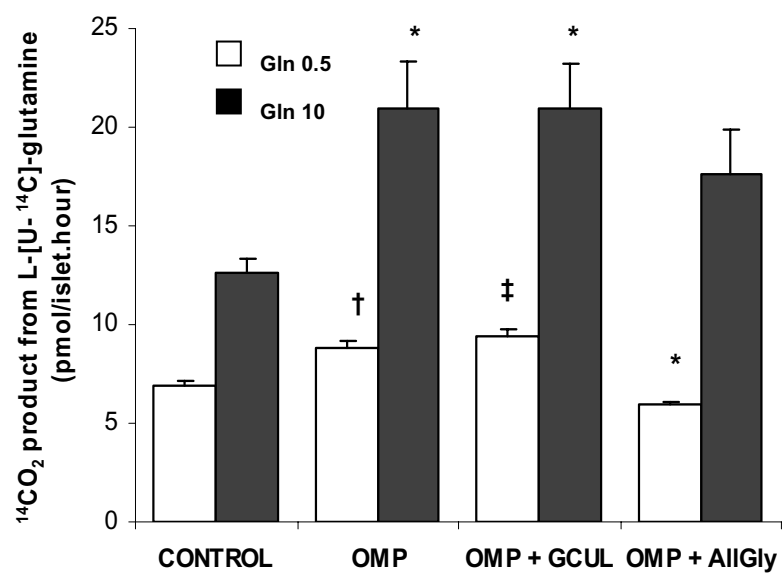


Fig 6

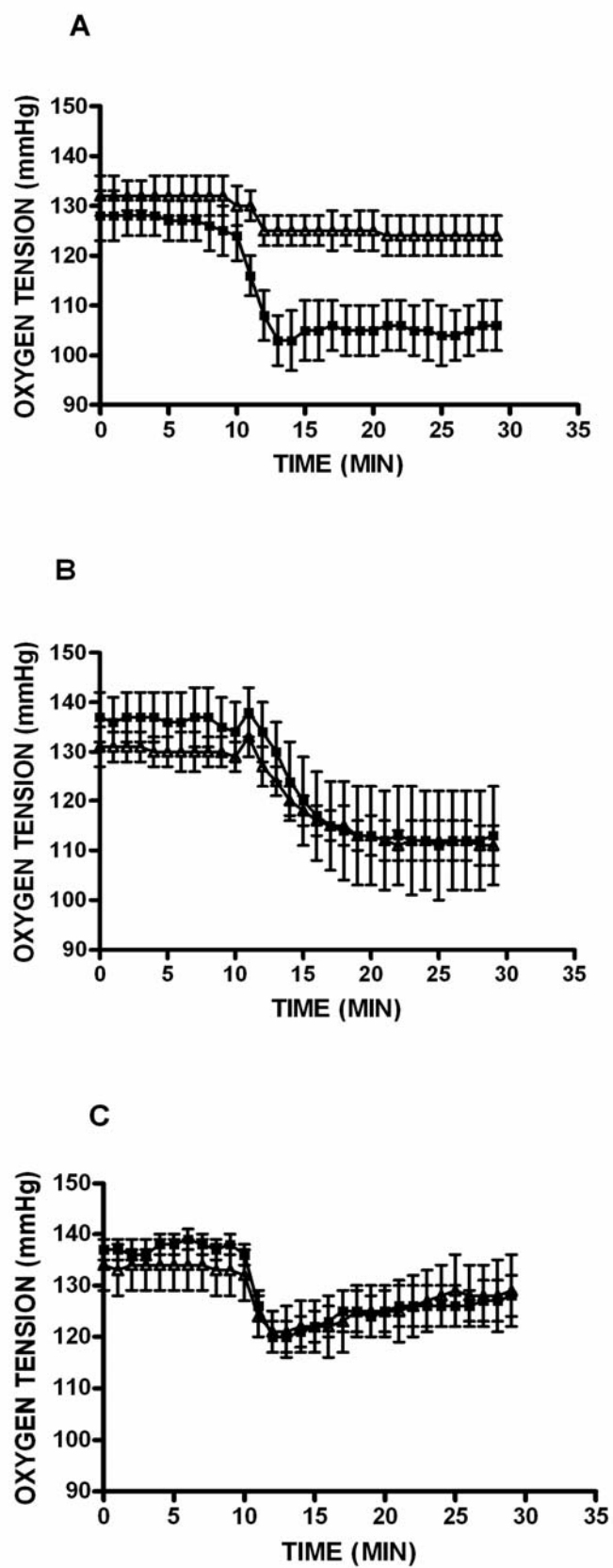


Fig. 7