Glutamate dehydrogenase contributes to leucine sensing in the regulation of autophagy

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Introduction

(Macro)autophagy is responsible for the degradation of long-lived proteins and for the elimination of either damaged or functionally redundant intracellular structures under stress conditions.1 In starvation, amino acids produced by autophagic proteolysis are recycled and used, at least in part, for the production of ATP to maintain cell survival. Amino acids are feedback inhibitors of autophagy so that autophagic flux decreases with increasing amino acid concentration.

Ever since the discovery that amino acids inhibit autophagy, at least in part by their ability to stimulate MTOR-mediated signaling,2 evidence is presented showing that glutamate dehydrogenase, the central enzyme in amino acid catabolism, contributes to leucine sensing in the regulation of autophagy. The data suggest a dual mechanism by which glutamate dehydrogenase activity modulates autophagy, i.e., by activating MTORC1 and by limiting the formation of reactive oxygen species.

Amino acids, leucine in particular, are known to inhibit autophagy, at least in part by their ability to stimulate MTOR-mediated signaling. Evidence is presented showing that glutamate dehydrogenase, the central enzyme in amino acid catabolism, contributes to leucine sensing in the regulation of autophagy. The data suggest a dual mechanism by which glutamate dehydrogenase activity modulates autophagy, i.e., by activating MTORC1 and by limiting the formation of reactive oxygen species.

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ascribed to stimulation of GLUD. Moreover, a combination of glutamine (a glutamate donor) and leucine, which maximizes the flux through GLUD, is most effective in stimulating phosphorylation of RPS6KB/S6K in β-cells and hepatocytes, and in inhibiting autophagic proteolysis in hepatocytes. Jukkat cells and HeLa cells.

In the present study, we describe experiments with HeLa cells in which GLUD1, the major isoenzyme, was knocked down. The data strongly suggest that GLUD1 does, indeed, contribute to the control of autophagy by amino acids, especially leucine. In addition, we showed that GLUD1, at least in part, controls autophagy by virtue of its ability to modulate MTOR signaling and to protect against the production of reactive oxygen species (ROS), known to be involved in the initiation of autophagy.

Results

Knockdown of GLUD1 promotes autophagy and inhibits MTORC1 activity. To evaluate a potential role for GLUD1 in the regulation of autophagy, we employed an RNA interference approach in HeLa cells, resulting in 80% to 90% reduction of GLUD1 protein (Fig. 1A). We then assessed the effect of GLUD1 knockdown on the conversion of cytosolic LC3-I to the lipid-bound LC3-II upon autophagosome formation. The amount of LC3-II reflects the number of autophagosomes and is widely accepted as a reliable measurement of autophagic flux. Because the steady-state level of autophagosomes is dependent on both “de novo” synthesis and consumption in the lysosomal compartment, the effects on autophagic flux are best determined in the presence of bafilomycin A1, which blocks the fusion and subsequent content degradation of autophagosomes in the lysosome. Silencing GLUD1 led to a significant increase of LC3-II, both in the presence and absence of bafilomycin A1 (Fig. 1A). In agreement, we observed diminished levels of the autophagy cargo protein SQSTM1/p62 in GLUD1-depleted cells, indicative of increased autophagic degradation (Fig. 1B). We next evaluated the effect of GLUD1 depletion on cellular LC3 distribution in HeLa cells stably transfected with GFP-LC3. In line with the results obtained with western blot analysis, GLUD1 knockdown led to a marked increase in GFP-positive punctate structures compared with control cells (Fig. 1C), indicating that GLUD1 is involved in the regulation of autophagy.

Because MTORC1 is the main repressor of autophagy under nutrient-rich conditions, we examined whether the increase in autophagy observed upon GLUD1 depletion was due to compromised MTORC1 activity. Indeed, silencing of GLUD1 reduced the phosphorylation of RPS6/S6 and EIF4EBP1/4E-BP1 (Fig. 1D), two direct downstream substrates of MTORC1, indicating that GLUD1 negatively regulates autophagy, at least in part, through modulation of MTORC1 activity.

Leucine inhibits autophagy in a GLUD1-dependent fashion. The activity of GLUD1 is specifically regulated by the amino acid leucine, which has been extensively studied as a potent suppressor of autophagy in many different cell types. In the first series of experiments, we confirmed the inhibitory effect of leucine on starvation-induced autophagy (Fig. 2). Leucine inhibited accumulation of LC3-II in a concentration-dependent manner upon nutrient starvation (Fig. 2A). Similarly, leucine prevented the accumulation of GFP-LC3 punctate structures (Fig. 2B). To verify that the inhibitory effect of leucine on autophagy was specific, we repeated the starvation experiments in the presence of the amino acid valine. The inhibitory effect of leucine on autophagy upon nutrient starvation could not be recapitulated by valine, as judged by the degree of LC3-II accumulation (Fig. 2A) and of GFP-LC3 puncta (Fig. 2B). From these results, we concluded that leucine indeed strongly inhibits starvation-induced formation of autophagosomes.

We next investigated whether the regulation of autophagy by leucine is dependent on the expression of GLUD1. For this purpose, we assessed the effect of leucine on autophagy in both control cells and in GLUD1-depleted cells (Fig. 3). Again, in nutrient-starved control cells, the presence of leucine was sufficient to inhibit the biosynthesis of autophagosomes as judged by the number of GFP-LC3 puncta (Fig. 3A). In agreement, leucine normalized the autophagic flux to the level observed in cells cultured in complete medium, as shown by the absence of LC3-II accumulation (Fig. 3B) and impaired degradation of SQSTM1 (Fig. 3C). Finally, leucine also prevented starvation-induced long-lived protein degradation (Fig. 3D). Importantly, we found that knockdown of GLUD1 abolished the inhibitory effect of leucine on the accumulation of GFP-LC3 puncta (Fig. 3A). The increase in autophagosomes correlated with a sustained autophagic flux and lysosomal activity as determined by the accumulation of LC3-II in bafilomycin A1-treated cells (Fig. 3B) and by the degradation of SQSTM1 (Fig. 3C), which could no longer be suppressed by leucine in GLUD1-depleted cells. Similarly, the rate of starvation-induced long-lived protein degradation in GLUD1-depleted cells was shown to be insensitive to leucine addition, but remained sensitive to 3-MA (Fig. 3D), an inhibitor of autophagosome formation by interfering with the activity of PI3K3 (the class III PtdIns3K). Taken together, these results strongly suggest that GLUD1 is required for the inhibitory effect of leucine on autophagy.

Leucine requires GLUD1 for promoting MTORC1 activity. Of all amino acids, leucine is not only the most effective in inhibiting autophagy but it can also stimulate MTORC1. Because leucine apparently requires GLUD1 in order to repress autophagy, we addressed whether this was due to its ability to reactivate the MTORC1 pathway. Here we showed that the presence of leucine, at least in part, preserved MTORC1 activation in starved control cells (Fig. 4). However, this stimulatory effect was abolished upon GLUD1 knockdown. Valine, which does not control autophagy, was not able to stimulate the MTORC1 pathway in either condition. These results suggest that leucine inhibits autophagy, at least in part, by activating MTORC1 in a GLUD1-dependent fashion.

Enhanced ROS production upon GLUD1-depletion contributes to autophagy induction. In addition to the gatekeeper function of MTORC1 in the regulation of autophagy, there is ample evidence suggesting that the intracellular levels of ROS may play a key role in the initiation of autophagy. Because GLUD1 activity results in the production of reducing equivalents, we
evaluated whether GLUD1, in addition to its ability to promote MTORC1 activity, could also influence the formation of ROS. Initial experiments with the ROS probe DHE indicated that ROS production was higher in GLUD1-depleted cells (Fig. 5A, left). Since DHE mainly detects cytosolic superoxide production, further experiments were performed with MitoSOX, the mitochondrial targeted form of DHE. The results presented in Figure 5A (right panel) show that mitochondrial ROS (mROS) production was also higher in GLUD1-depleted cells. These data indicate that flux through GLUD1 indeed contributes to the lowering of mROS production.

Furthermore, cytosolic and mitochondrial ROS were measured in the presence or absence of amino acids to determine whether the GLUD1-dependent inhibitory effect of amino acids on autophagy was mediated by ROS. As shown in Figure 5B and C, a complete mixture of amino acids significantly lowered cytosolic ROS and mROS production in control cells, but not in GLUD1-depleted cells. Also the relative effect of the combination of leucine and glutamine (to maximize flux through GLUD1) on ROS production was much lower in the GLUD1-depleted cells (Fig. 5C). Taken together, these data indicate that amino acids, and especially leucine, lower mROS by enhancing the flux through GLUD1.

To determine whether the elevated level of mROS observed in GLUD1-depleted cells contributes to the increased autophagosome formation, we performed short-term starvation experiments in the presence or absence of the general ROS scavenger N-acetylcysteine (NAC) and the mitochondrial specific ROS scavenger MitoQ. Addition of these scavengers to control cells slightly affected the degree of LC3-II accumulation (Fig. 5D). In contrast, the observed increase in autophagosome formation in GLUD1 knockdown cells was highly sensitive to both scavengers (Fig. 5D), indicating that the increased production of ROS upon GLUD1 depletion promotes the initiation of autophagy. These results are in line with a very recent report by Li et al., in which it is shown that NAC stimulates MTOR activity.

Discussion

Collectively, our data suggest a dual mechanism by which GLUD1 flux modulates autophagy, both by relaying cellular amino acid availability to MTORC1 and by generating reducing equivalents that interfere with the accumulation of ROS. Involvement of GLUD in amino acid sensing provides an explanation for the leucine specificity in the control of both processes, because this amino acid acts as an allosteric activator of GLUD.

Although the enzyme catalyzes a reversible reaction, the occurrence of hyperammonemia in hyperinsulinism/hyperammonia syndrome, when GLUD is overactivated, suggests that in vivo the enzyme operates in the direction of deamination of glutamate.

A possible connection between GLUD and the regulation of autophagy and MTOR may be related to NADPH, one of its reaction products. The mitochondrial respiratory chain is an important producer of ROS and can potentially harm the cell by oxidizing lipid, DNA and proteins. Autophagy can protect against these deleterious effects. There is ample evidence that autophagy is initiated by ROS in a manner that is sensitive to antioxidants and amino acids, perhaps because of the oxidation of a critical cysteine residue in ATG4. NADPH, derived from the GLUD reaction, may be used to scavenge ROS through, e.g., the glutathione-glutathione reductase system, and/or to prevent ROS production at complex I of the mitochondrial respiratory chain. It should be pointed out that this mechanism is MTORC1-independent and accounts for the disparity between the degree of MTORC1 activation and inhibition of autophagy (Figs. 3 and 4). This is in agreement with the fact that in the presence of amino acids, complete inhibition of MTORC1 by rapamycin never fully restores autophagic activity (see ref. 1 for a review).

GLUD can react with both NADP+ and NAD+ and the rate of their utilization is kinetically determined by their concentrations in the mitochondria. In case the enzyme would react with NAD+, NADPH can still be formed by transfer of hydrogen from NADH to NADP+, catalyzed by the energy-linked nicotinamide nucleotide transhydrogenase, which then also results in suppression of mitochondrial ROS production. The data of Figure 5 appear to support the view that GLUD can protect against ROS and in this way can contribute to the control of autophagy.

Another mechanism by which GLUD may contribute to amino acid sensing is related to the production of 2-oxoglutarate which, upon further metabolism, produces GTP in the succinylCoA synthetase reaction. This GTP may then be used to activate RHEB (Ras homolog enriched in brain) and RRAG, which would lead to autophagy inhibition, as hypothesized previously.

Involvement of GLUD in amino acid sensing may have its implications for the interpretation of some data on autophagy published in the past. For example, autophagy has been shown to be stimulated pharmacologically by the green tea component epigallocatechin gallate, a compound which, in addition to having other effects, is also a powerful inhibitor of GLUD. Chloroquine, often used in autophagic flux measurements because of its ability to raise the intralysosomal pH, is also
Figure 2. Leucine, not valine, inhibits autophagy. (A) Immunoblot analysis of LC3-I and LC3-II levels in HeLa cells cultured in complete medium or EBSS supplemented with 1, 5 or 10 mM of leucine or valine for 4 h. Immunoblotting of ACTB was used as a loading control. The LC3-II/ACTB ratio was determined using Bio-1D quantification software. Columns: mean; bars: SEM (n = 3); *p < 0.05. (B) Representative images of GFP-LC3 staining in HeLa GFP-LC3 cells cultured in complete medium or EBSS supplemented with 10 mM of leucine or valine for 4 h. The number of GFP-LC3 dots per cell was scored on 100 cells. Columns: mean; bars: SEM (n = 3); *p < 0.05.

Figure 3 (See opposite page). Knockdown of GLUD1 prevents the inhibition of autophagy by leucine. (A) The number of GFP-LC3 dots per cell was scored on 50 to 100 cells following a 72 h transfection with control (ct) siRNA (upper histogram) or GLUD1 siRNA (lower histogram) and a 4 h treatment with complete medium (CM), starved medium (EBSS) and starved medium supplemented with 10 mM leucine (EBSS+Leu). Columns: mean; bars: SEM (n = 3); **p < 0.01. (B) Immunoblot analysis of LC3-I and LC3-II levels in HeLa cells transfected with control (ct) siRNA or GLUD1 siRNA for 72 h and cultured in EBSS supplemented with 10 mM leucine or valine for 4 h. Where indicated, 200 nM of bafilomycin A1 (BAF) was present for 2 h to block the lysosomal degradation of LC3-II. Immunoblotting of ACTB was used as a loading control. The LC3-II/ACTB ratio was determined using Bio-1D quantification software. Columns: mean; bars: SEM (n = 3); **p < 0.01. (C) Immunoblot analysis of SQSTM1 levels in HeLa cells transfected with control (ct) siRNA (upper histogram) or GLUD1 siRNA (lower histogram) and cultured for 4 h in complete medium or EBSS supplemented with 10 mM leucine. Immunoblotting of ACTB was used as a loading control. The SQSTM1/ACTB ratio was determined using Bio-1D quantification software. Columns: mean; bars: SEM (n = 3); **p < 0.01. (D) Measurement of the degradation of long-lived proteins degradation in HeLa cells transfected with control (ct) siRNA (upper western blot and histogram) or GLUD1 siRNA (lower western blot and histogram) for 72 h and cultured in complete medium or EBSS supplemented with 10 mM leucine or 10 mM 3-methyladenine (3-MA). Columns: mean; bars: SEM (n = 3); *p < 0.05.
Figure 3. For figure legend, see page 854.
known to inhibit GLUD. This may lead to overestimation of the autophagic flux.

The interpretation of our data on GLUD is not compatible with previous studies on redox regulation of MTOR activity in which chemical compounds were used to manipulate the cellular redox state. For example, British anti-Lewisite (BAL), a strong reducing agent, inhibits, rather than stimulates, MTOR. However, it must be stressed that the effect of BAL on MTOR activity is probably unrelated to the physiological redox regulation of MTOR activity, because this compound inhibits mitochondrial electron transport between cytochrome b and c. The possibility that AMPK (which stimulates autophagy) is activated under these conditions cannot be ruled out.

Figure 4. Knockdown of GLUD1 prevents the stimulation of MTORC1 signaling by leucine. Immunoblot analysis of phospho-EIF4EBP1 (P-EIF4EBP1), EIF4EBP1, phospho-RPS6 (P-RPS6) and RPS6 in HeLa cells transfected with control (ct) siRNA or GLUD1 siRNA and cultured in complete medium or EBSS supplemented with 10 mM leucine or valine for 4 h. Columns: mean; bars: SEM (n = 3); *p < 0.05.

Figure 5 (See opposite page). Flux through GLUD1 inhibits induction of autophagy by promoting the elimination of ROS. (A) Cellular ROS production in HeLa cells transfected with control (ct) siRNA or GLUD1 siRNA was measured with DHE as probe as described in Materials and Methods (left histogram). Mitochondrial ROS production in HeLa cells transfected with control (ct) siRNA or GLUD1 siRNA was measured with MitoSOX as probe as described in Materials and Methods (right histogram). Columns: mean; bars: SEM (n = 3); *p < 0.05. (B) Cellular ROS production in HeLa cells transfected with control (ct) siRNA (left histogram) or GLUD1 siRNA (right histogram) was measured with DHE as probe as described in Materials and Methods. Cells were incubated in the absence (dotted lines) or presence (continuous lines) of amino acids for 45 min after which DHE was added for another 15 min. (C) Mitochondrial ROS production in HeLa cells transfected with control (ct) siRNA or GLUD1 siRNA was measured with MitoSOX as probe as described in Materials and Methods. The extent of MitoSOX oxidation was determined after 60 min of incubation with the dye. Columns: mean; bars: SEM (n = 8); **p < 0.01. Background fluorescence at t = 0 amounted to MFI = 13.3 ± 0.5. (D) Immunoblot analysis of LC3-I and LC3-II levels in HeLa cells transfected with control (ct) siRNA or GLUD1 siRNA for 72 h and cultured in EBSS supplemented with 10 mM N-acetylcysteine (NAC) or 0.5 μM MitoQ for 24 h. Where indicated, 200 nM of bafilomycin A1 (BAF) was present for 2 h to block the lysosomal degradation of LC3-II. Immunoblotting of ACTB was used as a loading control. The LC3-II/AcTB ratio was determined using Bio-1D quantification software. Columns: mean; bars: SEM (n = 3); **p < 0.01.
A

B

C

D

NAC | MitoQ | BAF
---|---|---
ct siRNA | - | - | + | + | - | -
GLUD1 siRNA | - | - | + | + | + | +

Quantification LC3-II/ACTB (a.u.)

NAC | MitoQ
---|---
- | -
+ | +

Quantification LC3-II/ACTB (a.u.)

NAC | MitoQ
---|---
- | -
+ | +

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As mentioned in the Introduction, recent information indicates that the v-ATPase in the lysosomal membrane acts as an amino acid sensor and that it responds to the level of amino acids within the lysosomal lumen. This level is determined by the amino acid sensor and that it responds to the level of amino acids.

In some of our experiments, we have used the v-ATPase inhibitor bafilomycin A₁ to monitor the appearance of LC3-II as a measure of autophagic flux, as recommended in reference 32. Because inhibition of the v-ATPase may have interfered with the amino acid-sensing mechanism as proposed previously, and thus with the autophagic process itself, we have checked its effect on MTOR activity in control experiments. However, in the presence of amino acids, we failed to observe an effect of bafilomycin A₁ on MTOR activity as measured by the phosphorylation of RPS6KB and EIF4EBP1 (data not shown). These results differ from the results obtained with concanamycin A and salicylhalamide A, two other v-ATPase inhibitors, but at least indicate that bafilomycin A₁, in our study for some reason had not interfered with the v-ATPase amino acid-sensing mechanism proposed by Zoncu et al.

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In our studies we have used starvation to induce autophagy. Of course it remains to be seen whether other stimuli of autophagy, also acting through MTOR, such as hypoxia and ER stress, will be similarly affected by leucine addition and GLUD depletion. Future studies may answer this question.

Upon completion of the present study, an article by Duran et al. was published, showing that metabolism of glutamine, through glutaminase and GLUD, stimulates MTOR signaling by enhancing GTP-loading of RRAG and reduces the steady-state level of autophagosomes within the cell (as predicted in refs. 1, 16, 17). The effect of leucine in this system could be ascribed to an activation of GLUD. Concomitant silencing of both glutaminase and GLUD blocked the ability of glutamine and of leucine to decrease the steady-state level of autophagosomes. Our results on the role of GLUD in the regulation of autophagic flux and MTOR signaling are in line with the data of Duran et al., and further extend their observations by demonstrating that flux through GLUD1 exerts control over autophagy by limiting the accumulation of ROS.

Materials and Methods

Reagents and antibodies. For cell treatments, bafilomycin A₁, 3-methyladenine (M9281), N-acetylcyesteine (A7250), leucine (L8912) and valine (V0500) were all purchased from Sigma. MitoQ [[10-(4,5-Dimethoxy-2-methyl-3,6-dioxo-cyclohexa-1,4-dienyl)-decyl]-triphenyl-phosphonium methanesulfonate] was a kind gift of Dr. M.P. Murphy (MRC, Cambridge, UK). Dyes used for ROS detection were dihydroethidium (DHE) and MitoSOX, both purchased from Invitrogen (D1168 and M36008, respectively). The following antibodies were used for immunoblotting: anti-LC3B (Sigma, L7543), anti-SQSTM1 (BD Biosciences, 610833), anti-β-ACTB/β-actin (Millipore, MAB1501), anti-phospho-EIF4EBP1/4EBP (Cell Signaling, 9459), anti-EIF4EBP (Cell Signaling, 9452), anti-phospho-RPS6/ S6 ribosomal protein (Cell Signaling, 2211), anti-RPS6 ribosomal protein (Cell Signaling, 2317), anti-mouse IgG-HRP (Bio-Rad, 170-6516), anti-rabbit IgG-HRP (Amersham, NA9340V).

Cell culture. The human cervical cancer cell line HeLa was grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C under 5% CO₂. The HeLa-GFP-LC3 cell line was obtained from Dr. Aviv M. Tolkovsky (University of Cambridge, UK), and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 500 μg/ml genitin at 37°C under 5% CO₂. For serum and amino acid deprivation, cells were cultured in Earle’s Balanced Salt Solution, fortified with 25 mM HEPES, pH 7.4. To test the effect of leucine and valine on autophagy, cells were cultured in Earle’s Balanced Salt Solution with 25 mM HEPES plus 10 mM leucine or 10 mM valine, unless otherwise indicated.

GFP-LC3 assay. The assay was performed in HeLa cells stably transfected with rat GFP-LC3. Prior to analysis, cells were treated as described in the text. Autophagy was then measured using a confocal microscope (LSM 510 Zeiss) by counting the number of GFP-LC3 puncta per cell with Imaris 6.3 software. At least 50 to 100 cells were counted per experiment.

Western blot analysis. Cellular extracts were prepared in 10 mM Tris pH 7.4, 1% SDS, 1 mM sodium vanadate, treated with benzonase (Sigma, E1014) for 5 min at room temperature, and boiled for 3 min. Fractions (20 μg) of the cellular extracts were subjected to SDS-PAGE. After electrophoresis, proteins were transferred to a protein nitrocellulose transfer membrane (Amersham Biosciences, RP303E). Before blocking membranes (with 5% nonfat milk protein in PBS/0.1% Tween-20), protein loading was assessed by Ponceau staining. Blots were then incubated with primary antibodies using the manufacturer’s protocol followed by the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG-HRP, Bio-Rad, 17-0516; anti-rabbit IgG-HRP, Amersham, NA9340V). Immunostained proteins were revealed using a chemiluminescent substrate (Millipore). Densitometric analysis on the immunoblots was quantified by Bio-1D software. The experiments were repeated at least three times, and representative autoradiograms are shown.

Analysis of degradation of long-lived proteins. This assay was adapted from Bauvy et al. 72 h after siRNA transfection, HeLa cells were incubated for 24 h at 37°C with 0.2 μCi/ml...
perform experiments in which small interfering RNAs against siRNA or GLUD1 siRNA were trypsinized and washed twice then incubated for 8 h at 37°C prior to the addition of 5% fetal using oligofectamine reagent (Invitrogen, 12252011). Cells were prepared from a 100× stock solution of non-essential AA (Gibco/glutamine (2 mM). A 25-fold concentrated AA mixture was pre-

**Transfections of cells with small interfering RNAs.**

In humans, there are two genes encoding GLUD: GLUD1 and GLUD2. GLUD1 occurs in all mammals but GLUD2 is only present in humans, where it is expressed in brain (astrocytes) and testis (Sertoli cells) only. For this reason, we decided to perform experiments in which small interfering RNAs against GLUD1 were used (Invitrogen). Control siRNA was synthesized by Eurogentec (Seraing, Belgium). Cells cultured in 6-well plates were transfected with siRNA at 200 nM final concentration by using oligofectamine reagent (Invitrogen, 12252011). Cells were then incubated for 8 h at 37°C prior to the addition of 5% fetal bovine serum and then left for another 72 h. Cells were then treated as described in the text and subjected to western blotting analysis, long-lived protein degradation or GFP-LC3 assays.

**Measurement of ROS production.**

Cells treated with control siRNA or GLUD1 siRNA were trypsinized and washed twice with incubation medium, consisting of 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1.2 mM KPi, 1 mM MgCl₂, 1.3 mM CaCl₂ and 0.2% (w/v) bovine serum albumin (final pH 7.4). Cells were resuspended at a concentration of 0.5 × 10⁶/ml and preincubated for 45 min at 37°C in the absence or presence of a complete amino acid (AA) mixture or with a combination of leucine (1 mM) and glutamine (2 mM). A 25-fold concentrated AA mixture was prepared from a 100× stock solution of non-essential AA (Gibco/Invitrogen, 11140) and a 50× stock solution of essential AA (Gibco/Invitrogen, 11130) with glutamine (20 mM) and HEPES (50 mM) added and sufficient NaOH to neutralize the pH of this acidic mixture. After the preincubation plus or minus AA, the cells (in 300 μl aliquots) were transferred to tubes containing 1 μl MitoSOX (500 μM, dissolved in DMSO) or, in subsequent experiments, to tubes containing 1 μl MitoSOX (300 μM, dissolved in DMSO). Samples were analyzed without washing on a FACScalibur (BD Biosciences) and results of 6000 events were expressed as mean fluorescence intensity (MFI) in the red (FL3) channel. The increase in red fluorescence with MitoSOX (a measure for mitochondrial superoxide production) proved to be linear over a period of at least 90 min. Cell debris and cells showing a very high red fluorescence (< 5% of the total population) were excluded from the analysis. High MitoSOX staining appeared to be due to binding to the DNA of dead cells as demonstrated in control experiments by double staining with Sytox Green (Invitrogen, S7020).

**Statistical analysis.** Data are expressed as means ± standard errors of the means (SEM) of at least three experiments. The statistical significance was assessed by a Student’s t-test. p < 0.05 was considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

There are no conflicts of interest.

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