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Serum starvation: *caveat emptor*

Sergej Pirkmajer and Alexander V. Chibalin

Department of Molecular Medicine and Surgery, Integrative Physiology, Karolinska Institutet, Stockholm, Sweden

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Pirkmajer S, Chibalin AV. Serum starvation: *caveat emptor*. *Am J Physiol Cell Physiol* 301: C272–C279, 2011. First published May 25, 2011; doi:10.1152/ajpcell.00091.2011.—Serum starvation is one of the most frequently performed procedures in molecular biology and there are literally thousands of research papers reporting its use. In fact, this method has become so ingrained in certain areas of research that reports often simply state that cells were serum starved without providing any factual details as to how the procedure was carried out. Even so, we quite obviously lack unequivocal terminology, standard protocols, and perhaps most surprisingly, a common conceptual basis when performing serum starvation. Such inconsistencies not only hinder interstudy comparability but can lead to opposing and inconsistent experimental results. Although it is frequently assumed that serum starvation reduces basal activity of cells, available experimental data do not entirely support this notion. To address this important issue, we studied primary human myotubes, rat L6 myotubes and human embryonic kidney (HEK)293 cells under different serum starvation conditions and followed time-dependent changes in important signaling pathways such as the extracellular signal-regulated kinase 1/2, the AMP-activated protein kinase, and the mammalian target of rapamycin. Serum starvation induced a swift and dynamic response, which displayed obvious qualitative and quantitative differences across different cell types and experimental conditions despite certain unifying features. There was no uniform reduction in basal signaling activity. Serum starvation clearly represents a major event that triggers a plethora of divergent responses and has therefore great potential to interfere with the experimental results and affect subsequent conclusions.

serum-free medium; basal phosphorylation; AMP-activated protein kinase; mammalian target of rapamycin; extracellular signal-regulated kinase 1/2

THE TERM SERUM STARVATION, or simply starvation, as well as serum deprivation, depletion, removal, restriction, withdrawal, and serum limitation have been used to denote various procedures that include growing cells in either reduced serum, serum-free, or serum- and protein-free medium (5, 14, 16, 27, 28, 34, 45, 47, 50, 53, 56, 57, 70). Unfortunately, none of these terms have a well-defined meaning that would unambiguously reflect the actual procedure, and they have all been inconsistently applied to describe different protocols. It is common, but by no means universal, to perform serum starvation in serum-free basal medium with or without bovine serum albumin (BSA) (11, 23, 28, 37, 57, 58, 68, 71). Sometimes specific growth factors and hormones are added to serum-free starvation medium (50). Alternatively, serum starvation may be carried out in medium with low serum concentration (relative to normal growth medium), typically 0.1–0.5% (6, 15, 36, 47, 60), but sometimes as high as 2–5% (13, 19, 59) or as low as 0.05% (32, 58). The time frame for serum starvation is equally varied and can include anything from 15 to 30 min up to several weeks (1, 3, 4, 27, 28, 49, 58, 63, 72). Moreover, while alternative designations such as serum withdrawal or deprivation are sometimes used synonymously and interchangeably with serum starvation (27, 34, 56, 72), others make a clear distinction based on serum concentration (15). We will not attempt to redefine these terms and incubating cells in reduced serum or serum-free medium in all its different forms will be laxly referred to as serum starvation in this paper.

CONCEPTUAL DIFFERENCES

Based on the rationale for performing serum starvation, at least three groups of studies can be identified. Serum starvation is often, at least implicitly, regarded as a routine procedure carried out to prepare cells for an experiment in serum-free conditions and therefore not an experiment per se (5, 9, 37, 54). Although serum provides optimal conditions for cell growth, its poorly defined complex and above all variable composition represents an important and undesirable confounding factor while performing bioassays (30, 40, 62, 75). Elimination of serum from culture medium therefore removes known and unknown unknowns, reduces analytical interference, and provides more reproducible experimental conditions (17, 33, 42). Moreover, it (supposedly) reduces basal cellular activity (15) and makes the population of proliferating cells more homogenous, since they withdraw from the cell cycle to enter the quiescent G_0/G_1 phase (49, 63). Serum starvation-induced synchronization, followed by serum restimulation or preceded by serum shock (e.g., 50% serum), has been extensively used in cell cycle and circadian rhythm research ever since Pardee established the restriction point concept (4, 47, 72) and has remained an important and valuable experimental approach (29, 35, 41, 63) despite certain reservations (18). The usefulness of serum starvation goes beyond basic molecular biology and includes metabolic research, where the introduction of serum starvation-based protocols revealed the normal (physiological) response to insulin in cultured skeletal muscle cells, while earlier attempts to study molecular mechanisms of insulin action had been hampered by the presence of serum (12, 28).

In contrast, many researchers have used serum starvation as a tool to study molecular mechanisms involved in protein degradation (20, 26), cellular stress response (3, 36), autophagy, apoptosis (6, 60, 74), and/or to simulate particular pathological conditions (see the end of this paragraph). This is an obvious conceptual drift from the more technically oriented approach mentioned above, where these responses are seen as an undesirable side effect (17) and certainly not the reason for carrying out serum starvation. Even if these considerations are put aside, it is clear that under these circumstances serum starvation has now shifted from being a preparatory phase for the experiment to being an experiment in its own right ("cells were treated with serum starvation") (74). The same goes for studies where serum starvation in combination with hypoxia

Address for reprint requests and other correspondence: A. V. Chibalin, Dept. of Molecular Medicine and Surgery, Integrative Physiology, Karolinska Institutet, von Eulers väg 4, 4 tr, SE-171 77, Stockholm, Sweden (e-mail: Alexander.Chibalin@ki.se).

and/or lowered glucose content is used as an experimental model to mimic clinical conditions like myocardial infarction and stroke (7, 11, 25, 70) or to recreate a poorly vascularized, nutrient-, growth factor-, and oxygen-deficient core of tumors (36, 52, 59).

ELUSIVE REDUCTION IN BASAL ACTIVITY

It is often assumed that serum-starved cells have reduced (ill-defined) basal cellular activity (15), but serum starvation has also been referred to as "environmental stress" (38), "serum starvation stress" (36, 46), and "apoptotic trigger" (8), implying dynamism that does not entirely fit the idea of a passive entry into a dormant hypoactive state. Levin et al. (36), in a comprehensive and elegant proteomic study, clearly showed divergent responses to 24-h serum starvation in medium containing 0.5% fetal bovine serum (FBS) across different signaling pathways and tumor cell types. Whereas several aspects of signaling response to serum-free conditions have already been carefully studied (12, 53, 58), it remains an open issue whether diversity observed in the presence of serum (36) also exists when cells are serum starved in serum-free medium.

To systematically address the issue of time- and protocol-dependent changes in the basal signaling activity in different cell types, which are frequently used in molecular endocrinology and metabolism research (5, 12, 22, 28, 37, 54, 61), primary human myotubes, rat L6 myotubes, and human embryonic kidney (HEK)293 cells were serum starved for 24 h in serum-free medium. We assessed phosphorylation changes (Western blot) in the energy-sensing AMP-activated protein kinase (AMPK) and its downstream target acetyl-CoA carboxylase (ACC), the extracellular signal-regulated kinase (ERK1/2), and the mammalian target of rapamycin (mTOR) pathway.

HEK293 cells, grown on ordinary polystyrene or polylysine-coated six-well plates, were serum starved in the presence or absence of 0.5% (wt/vol) BSA. Polylysine-coated plates are very useful since they improve the adhesion of HEK293 cells, but different substrata are known to modulate cellular responsiveness to various stimuli (64, 68, 73) and could affect also the response to serum starvation. Ponceau staining was used to assess equal protein loading instead of internal protein control, since total protein stains have the advantage of not being dependent on the expression of a single housekeeping protein (2, 43, 51, 65). We decided not to use GAPDH since it displayed some (albeit limited) variation to serum starvation in a few cases (Fig. 1), but a detailed analysis of its stability and expression under serum-starved conditions was not performed.

AMPK, ACC, and ERK1/2 phosphorylation responded to serum starvation dynamically with time-dependent up- or downregulation (Fig. 2), which was clearly influenced by cell type and experimental conditions. Similar responses were observed by Levin et al. (36) who analyzed a large array of signaling molecules in adenocarcinoma and glioblastoma cell lines after 24-h serum starvation in 0.5% FBS. Contrary to our results in human and L6 myotubes (Fig. 2, *A* and *B*), an increase in AMPK and ACC phosphorylation has been previously reported for serum-starved C2C12 myotubes (31) and L6 myotubes (12). The discrepancy might be due to differences in media composition since C2C12 were grown in 10% bovine calf serum before serum starvation (31) and L6 myotubes in DMEM (12) rather than 2% FBS and MEM-α, respectively

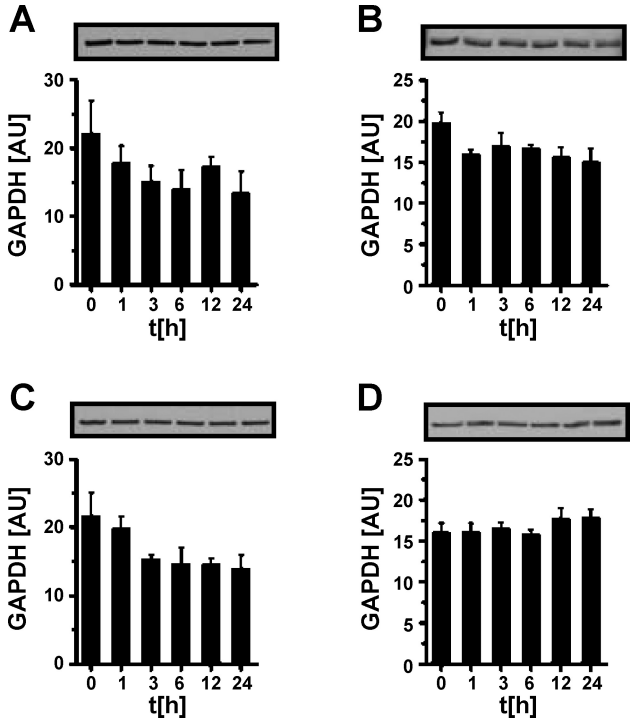

Fig. 1. GAPDH expression in serum-starved primary human myotubes, L6 myotubes and HEK293 cells. Treatments were as described in Fig. 2. *A:* primary human myotubes ($n = 4$). *B:* rat L6 myotubes ($n = 4$). *C:* HEK293 cells (polystyrene plates, without BSA) ($n \pm 4$). *D:* HEK293 cells (polystyrene plates, 0.5% (wt/vol) BSA) ($n = 8$). Columns are means ± SE. Changes in GAPDH expression level between serum-starved and nonstarved (serum containing) conditions ($t = 0$ h) were not statistically significant (one-way ANOVA, followed by Dunnett's post hoc test, $P > 0.05$). However, since a tendency toward a decrease in GAPDH expression was repeatedly seen (*A–C*), Ponceau staining was used for loading control instead. Detailed analysis of GAPDH expression under serum starvation was not carried out.

(see *Materials and Methods* in the APPENDIX). Unlike myotubes, serum-starved HEK293 cells showed at least a transient increase in AMPK phosphorylation compared with nonstarved controls (Fig. 2, *C–F*). However, AMPK and ACC phosphorylation did not always correlate in HEK293 cells (Fig. 2, *E* and *F*), which makes the interpretation of these changes difficult. Although ACC is a well-characterized AMPK target, discrepancies in the basal phosphorylation levels of these proteins have been noted previously (1).

Analogous to hepatoma cells (53), human myotubes responded to serum starvation with a pronounced increase in ERK1/2 phosphorylation (Fig. 2*A*). In L6 cells ERK1/2 phosphorylation partially recovered after initial profound dephosphorylation and rose to a transient relative peak after 6 h in serum-free conditions (Fig. 2*B*), which is strikingly similar to response in cardiac fibroblasts (34). In contrast, HEK293 cells displayed a relative or absolute peak in ERK1/2 phosphorylation at the end of 24-h starvation (Fig. 2, *C–F*). Comparable late ERK1/2 rephosphorylation was observed in murine embryonic fibroblasts starved in serum-free medium but not in the presence of 0.05% FBS (58). Waveform up- and downregulation in phospho-AMPK and phospho-ERK1/2 is reminiscent of circadian rhythms (4), and it is tempting to speculate that this might represent some kind of endogenous oscillation, perhaps with different periods in myotubes and HEK293 cells.

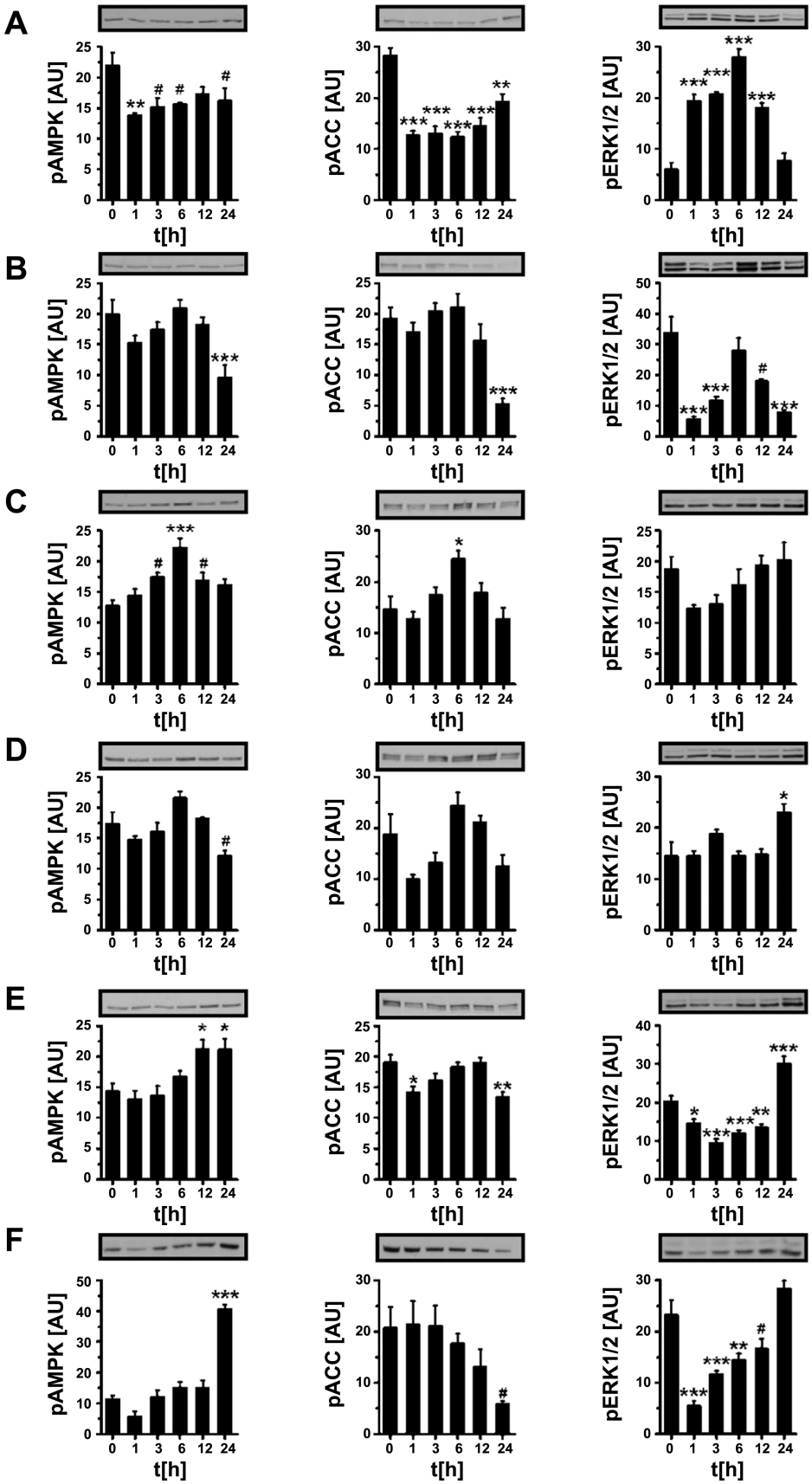

Fig. 2. Serum starvation-dependent changes in the basal phosphorylation of AMP-activated protein kinase (AMPK)α (T172), acetyl-CoA carboxylase (ACC) (S79), and extracellular signal-regulated kinase 1/2 (ERK1/)2 (T202/Y204). Primary human myotubes and rat L6 myotubes were serum starved for the indicated periods of time in serum-free DMEM (low glucose) or MEM-α, respectively. Human embryonic kidney (HEK)293 cells, grown on ordinary polystyrene or polylysine-coated plates, were serum starved in serum-free DMEM (high glucose) in the presence or absence of 0.5% (wt/vol) bovine serum albumin (BSA). The initial time point ($t = 0$ h) represents nonstarved condition, i.e., cells grown in the presence of 2% (vol/vol) fetal bovine serum (FBS) (primary human and L6 myotubes) or 10% (vol/vol) FBS (HEK293 cells). *A*: primary human myotubes ($N = 5$). *B*: rat L6 myotubes ($N = 4–8$). *C*: HEK293 cells (polystyrene plates, without BSA) ($N = 4–8$). *D*: HEK293 cells (polylysine-coated plates, without BSA) ($N = 4$). *E*: HEK293 cells [polystyrene plates, 0.5% (wt/vol) BSA] ($N = 8$). *F*: HEK293 cells [polylysine-coated plates, 0.5% (wt/vol) BSA] ($N = 5$). Columns are means ± SE. Dunnett's post hoc test for serum-starved vs. nonstarved (serum containing) condition ($t = 0$ h): #$P < 0.05$, *$P < 0.01$, **$P < 0.005$, and ***$P < 0.001$.

Protein synthesis and cell growth are regulated by mTOR pathway, which is in turn indirectly dependent on nutrient, growth factor, and energy availability. Signals through nutrient-sensing (e.g., Rag proteins), growth factor-sensing (e.g., ERK1/2, Akt), and energy-sensing (AMPK) pathways are channeled to mTOR complex (mTORC) 1 and 2, where diverse inputs are integrated and transduced into appropriate effector responses (10, 21, 39, 76). Key signaling events downstream of mTORC1 are the phosphorylation of 70 kDa ribosomal protein S6 kinase (p70S6K) and 4E-binding protein 1 (4EBP1). Whereas phosphorylation of p70S6K promotes protein synthesis through activation of ribosomal protein S6 (rpS6) and several other factors, phosphorylated 4EBP1 releases the bound eukaryotic initiation factor 4E (eIF-4E) and thereby disinhibits initiation of translation. Although rpS6 is a well-known downstream target of p70S6K, its exact role has recently become somewhat uncertain (10, 21, 24, 39, 76).

Serum starvation in serum-free medium led to a more or less uniform response in mTOR pathway (Fig. 3). The most conspicuous changes were substantial decrease in p70S6K and rpS6 phosphorylation, which was evident in all cases with the exception of an early and transient increase in human myotubes (Fig. 3*A*). Serum starvation of adenocarcinoma and glioblastoma cells has usually led to decreased p70S6K phosphorylation, but occasionally increases have been noted (36). Protein synthesis was not the subject of this study; however, in line with more or less uniform reduction in activity of the mTOR pathway, we noticed that total protein content (per well) tended to be lower in the serum-starved cells (data not shown) compared with the nonstarved controls. The observed reduction in total protein content is consistent with the serum starvation-induced decrease in protein synthesis and the concomitant increase in proteolysis (20, 26, 72). Since the mTOR pathway is subject to regulation by multiple upstream pathways that are affected by serum starvation in different ways and to variable degrees, it is not possible to explain all the observed trends only by observing AMPK and ERK1/2. Nevertheless, overall p70S6K and rpS6 phosphorylation appears to be at least partially dependent on relative levels of phospho-AMPK and phospho-ERK1/2 that inhibit or activate, respectively, the mTOR pathway.

Apart from L6 myotubes (Fig. 3*B*), 4EBP1 phosphorylation did not correlate well with changes in p70S6K-rpS6 phosphorylation, which may probably be explained by their differential roles in translation and cell growth regulation (10, 21). In one case 4EBP1 phosphorylation was substantially upregulated after 24-h serum starvation (Fig. 3*E*), which parallels increases in total 4EBP1 in serum-starved pancreatic β-cells (48). Phosphorylation of mTOR Ser^{2448} displayed a more subdued response with an overall tendency toward a decrease in phosphorylation (Fig. 3). In contrast, serum-starved carcinoma and glioblastoma cells responded either with no change or an increase in mTOR Ser^{2448} phosphorylation (36). It is interesting to observe that mTOR Ser^{2448}, which was assumed to be primarily an insulin-responsive site in the past, but is more probably a part of p70S6K-rpS6 feedback loop (10, 21), had the lowest phosphorylation level after 6 h of serum starvation (Fig. 3*E*), when p70S6K and rpS6 were almost completely dephosphorylated, and phospho-Akt (Fig. 4*A*) was near its peak. A similar increase in Akt phosphorylation was also seen in serum-starved hepatoma cells (53) and L6 myotubes (12).

Serum starvation is often used to study apoptosis and increased caspase 3 activity has been reported in serum-starved HEK293 cells (67). Nevertheless, caspase 3 was not activated in response to serum starvation neither in myotubes nor in HEK293 cells (Fig. 4). It seems that caspase 3 activation in response to serum starvation is, to a certain extent, a variable and time-dependent event. Levin et al. (36) have reported that caspase 3 activation might be increased, decreased, or not affected by 24-h serum starvation in 0.5% FBS, whereas others have noticed that apoptosis can be triggered in serum-starved cells without activating caspase 3 (34). In HEK293 cells caspase 3 activation was especially pronounced with more prolonged serum starvation (67).

CONCLUSIONS

Serum starvation elicited complex and unpredictable time-dependent and cell-type dependent effects. Neither short nor prolonged incubation in serum-free medium led to a uniform reduction in the basal activity of intracellular signaling pathways. While different signaling pathways showed diametrically opposing changes in the same cell type [cf. phospho-ERK1/2 and phospho-ACC in human myotubes or phospho-ERK1/2 and phospho-Akt (Fig. 4*B*) in HEK293 cells], some cascades (e.g., ERK1/2) displayed divergent, cell type-dependent responses. Signaling response across different cell types and experimental conditions displayed less variation in mTOR pathway, especially in p70S6K and rpS6 phosphorylation, which was always profoundly reduced after 24-h serum starvation (Fig. 3). These observations are consistent with earlier reports (12, 34, 36, 53, 58), but a unifying mechanistic explanation remains elusive. Although serum starvation-induced changes in the mTOR pathway can be related to growth factor deprivation and opposing effects of its upstream regulators like AMPK and ERK1/2, it is not immediately obvious why serum starvation causes changes in phosphorylation of AMPK. Since basal medium contains key macronutrients (glucose, amino acids), at least early events are probably not a reflection of disrupted energy metabolism. Moreover, there was no consistent change in AMPK phosphorylation in different cell types. Of course, nutrient availability is not synonymous with utilization, which could clearly be affected by pronounced changes in cellular environment. After all, untreated type 1 diabetic patients are in a profound starvation-like catabolic state despite having all essential nutrients at their disposal. Other possible reasons for dynamic signaling response(s) could be changes in the production of reactive oxygen species (53) or secretion and shedding (from cell surface) of various proteins involved in intercellular communication and other functions (42, 55). Taking existing reports into account, it does not seem probable that serum starvation-induced molecular events may be ascribed to one simple mechanism, and an interplay of different simultaneous synergistic or antagonistic inputs is much more likely.

It is equally obvious that when low basal activity of a particular signaling pathway is desirable for analytical reasons, the optimal time frame for serum starvation should be experimentally established and not simply based on the premise of uniform decrease in signaling. However, even if the best conditions from the analytical perspective are known, certain caveats beyond changes in basal activity and elimination of serum-dependent confounding factors should

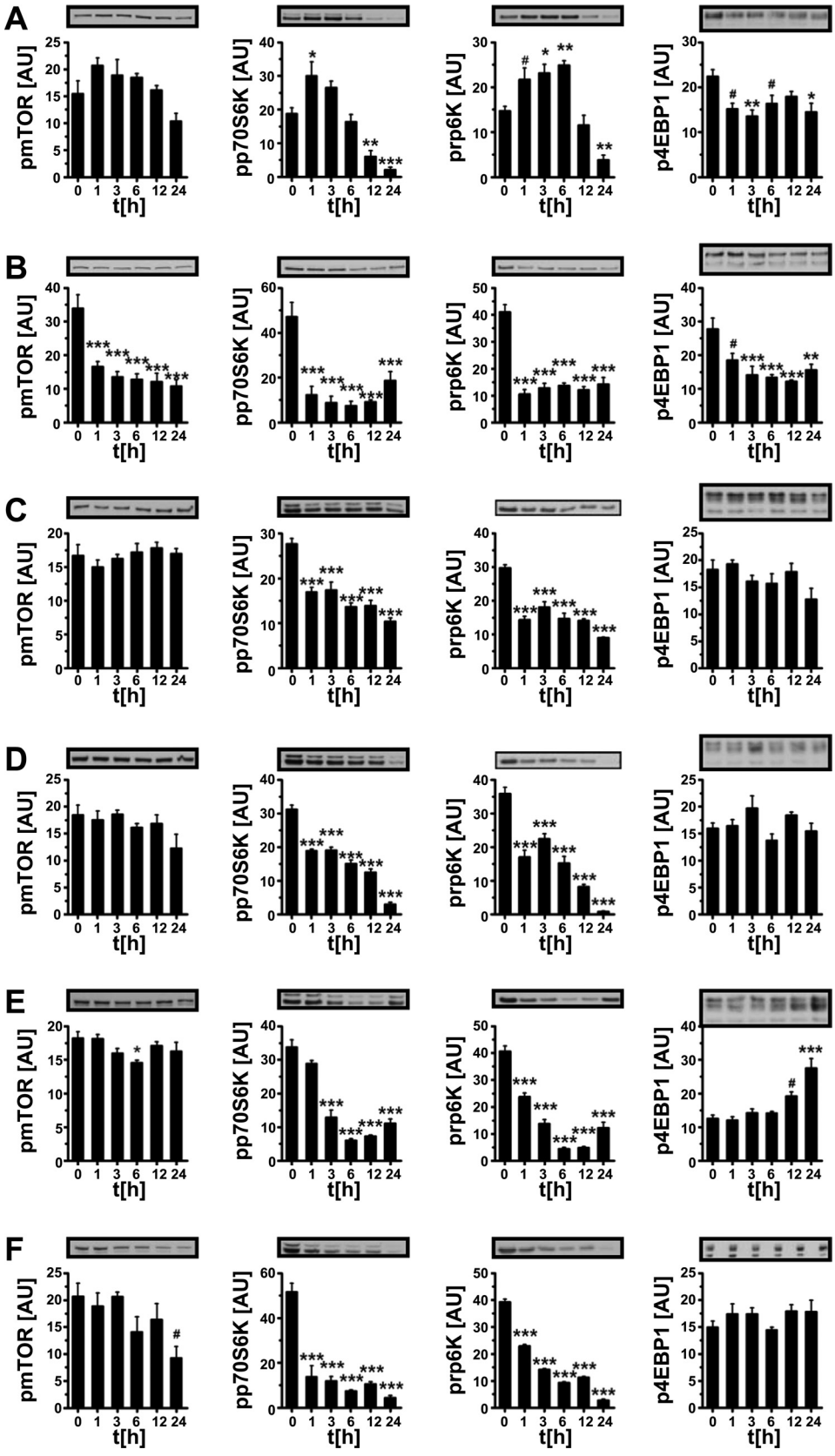

Fig. 3. Serum starvation-dependent changes in the basal phosphorylation of mammalian target of rapamycin (mTOR) (S2448), phosphorylation of 70 kDa ribosomal protein S6 kinase (p70S6K) (T389), ribosomal protein S6 (rpS6) (S235/236), and 4E-binding protein 1 (4EBP1) (T37/46). Treatments were as described in Fig. 2. *A*: primary human myotubes ($N = 4–5$). *B*: rat L6 myotubes ($N = 3–8$). *C*: HEK293 cells (polystyrene plates, without BSA) ($N = 8$). *D*: HEK293 cells (polylysine-coated plates, without BSA) ($N = 4$). *E*: HEK293 cells [polystyrene plates, 0.5% (wt/vol) BSA] ($N = 6–8$). *F*: HEK293 cells [polylysine-coated plates, 0.5% (wt/vol) BSA] ($N = 5$). Columns are means ± SE. Dunnett's post hoc test for serum starved vs. nonstarved (serum containing) condition ($t = 0$ h): #$P < 0.05$, *$P < 0.01$, **$P < 0.005$, and ***$P < 0.001$.

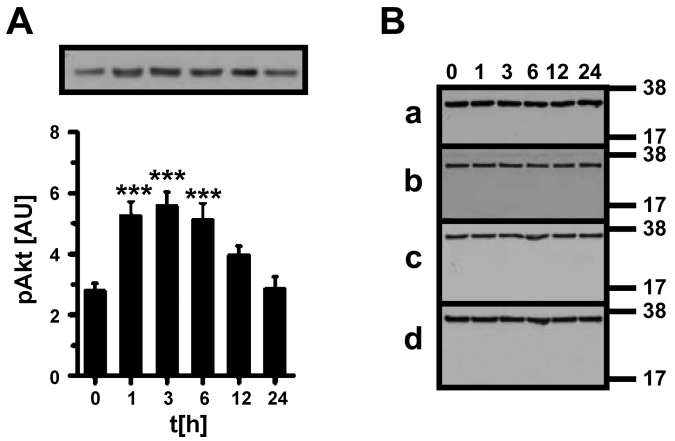

Fig. 4. Serum starvation-dependent changes in the basal level of phospho-Akt (S473) and (Pro)caspase 3. Primary human myotubes, rat L6 myotubes, and HEK293 cells were serum starved in serum-free medium as described in Fig. 2. *A:* phospho-Akt (pAkt) expression in serum-starved HEK293 cells [polystyrene plates, 0.5% (wt/vol) BSA) (n = 7–8). Columns are means ± SE. Dunnett's post hoc test for serum-starved vs. nonstarved (serum containing) condition: ***$P < 0.001$. *B:* procaspase 3 (~35 kDa) was clearly expressed in all cases, but no caspase 3 activation could be observed during the 24-h serum starvation. Primary human myotubes (*a*), rat L6 myotubes (*b*), HEK293 cells on polystyrene plates (without BSA) (*c*), and HEK293 cells on polylysine-coated plates (without BSA) (*d*). Approximate molecular masses are shown on the right (kDa). Incubation time in serum-free medium is indicated above the blots (in h).

be considered. Precision, consistency, and reproducibility do not equal accuracy, or for that matter, physiological relevance. Although serum starvation has proved to be very useful in cell cycle, circadian rhythm, molecular endocrinology, and metabolism research (4, 9, 12, 28, 29, 35, 41, 47, 54) and will undoubtedly remain indispensable laboratory procedure, physiological extrapolations of results obtained from serum-starved cells should be subject to constant scrutiny. The presence or absence of serum (or specific supplements) can influence cellular phenotypic characteristics (15, 27, 44, 69, 71), affect fold response to various stimuli (1, 66), or even completely change the result of an experiment (66). One would therefore be well advised not to avoid confounding Scylla only to be engulfed by serum-starved Charybdis.

APPENDIX

The inclusion of the following *Materials and Methods* is provided as an aid to the readers.

Materials and Methods

Antibodies and reagents. Phosphospecific antibodies against pERK1/2 (T202/Y204), pAMPKα (T172), pACC (S79), pp70S6K (T389), prpS6 (S235/236), p4EBP1 (T37/46), and pAkt (S473) and the Caspase 3 antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-mTOR (S2448) was purchased from Rockland (Gilbertsville, PA). Horseradish peroxidase-conjugated goat anti-rabbit antibody, MES running buffer, and resolving 4–12% bis-Tris gels were obtained from Bio-Rad (Richmond, CA). ECL reagent and protein molecular weight marker (full range rainbow marker) were purchased from GE Healthcare (Uppsala, Sweden). BCA Protein Assay kit was from Pierce (Rockford, IL), Protease Inhibitor Cocktail from Calbiochem (La Jolla, CA), and polyvinyl difluoride (PVDF) Immobilon-P membrane from Millipore (Bedford, MA). Cell culture media and reagents were from Invitrogen (Stockholm, Sweden). Cell culture flasks and plates were from Costar (Nordic Biolabs, Täby, Sweden), TPP and Becton & Dickinson. All other reagents, unless otherwise specified, were obtained from Sigma-Aldrich (Stockholm, Sweden).

Cell culture and serum starvation protocol. Human skeletal muscle cells were grown and differentiated into myotubes as previously described (1). On the seventh day of differentiation serum containing differentiation medium [low glucose DMEM supplemented with 2% (vol/vol) FBS, 1% (vol/vol) penicillin-streptomycin and 1% (vol/vol) Fungizone] was removed, and cells were washed twice with warm sterile phosphate-buffered saline (PBS). Human myotubes were serum starved in serum-free DMEM without antibiotics and antimycotics for up to 24 h (see Fig. 2). L6 cells (provided by Dr. Amira Klip, The Hospital for Sick Children, Toronto, ON, Canada) were grown and differentiated into myotubes as previously described (5). On the seventh day of differentiation, serum-containing differentiation medium [MEM-α supplemented with 2% (vol/vol) FBS, 1% (vol/vol) penicillin-streptomycin, 1% (vol/vol) Fungizone] was removed, and cells were washed twice with warm sterile PBS. L6 myotubes were serum starved in serum-free MEM-α without antibiotics and antimycotics for up to 24 h (see Fig. 2). Experiments on human and L6 myotubes were carried out in polystyrene six-well plates. HEK293 cells were grown in high-glucose (4,500 mg/l) DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin but without Fungizone. Twenty four to forty eight hours before experiment they were seeded either in polystyrene or polylysine-coated six-well plates (Beckton & Dickinson). On the day of experiment, serum-containing medium was removed, and cells were carefully washed twice with warm sterile PBS. HEK293 cells were serum starved in serum-free high-glucose DMEM in the presence or absence of 0.5% (wt/vol) bovine serum albumin (BSA) for up to 24 h (see Fig. 2).

Western blot. Cells were washed three times with ice-cold PBS at the end of experiment and then lyzed in 150–200 μl homogenization buffer [1% (vol/vol) Protease Inhibitor Cocktail, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_3VO_4, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 20 mM Tris, 10 mM NaF, 1 mM EDTA, and 1 mM PMSF]. Lysates were centrifuged at 4°C (12,000 *g,* 15 min), supernatants were collected, and total protein was measured with the BCA Protein Assay. Samples were diluted with homogenization buffer to the same final protein concentration, prepared in Laemmli buffer for SDS-PAGE, resolved on 4–12% bis-Tris gel, and then transferred to PVDF membrane. After transfer, membranes were quickly washed in 5% (vol/vol) acetic acid and stained with 0.1% (wt/vol) Ponceau S in 5% (vol/vol) acetic acid. Ponceau staining was used for quality control of transfer procedure and to assess equal sample loading (see below). A scan of the stained membrane was taken in all cases. This was followed by washing in TBS-T [10 mM Tris, 137 mM NaCl, 0.02% (vol/vol) Tween-20, pH 7.6] and blocking in 7.5% (wt/vol) non-fat milk in TBS-T. Membranes were then incubated with the appropriate primary antibody overnight at 4°C, which was followed by washing in TBS-T and a 1-h incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized by enhanced chemiluminiscence and quantified by Quantity One 1-D Analysis Software (Bio-Rad). Total protein staining is an alternative to using internal protein control (2, 43, 51, 65). Ponceau-stained bands (4–6 per lane) on every PVDF membrane were therefore densitometrically quantified to assess equal protein loading (51).

Statistics. Data are presented as means ± SE. One-way ANOVA followed by either Bonferroni's or Dunnett's post hoc test was performed to test for significant differences. Differences were considered statistically significant at $P < 0.05$. Statistical analysis was carried out with GraphPad Prism 5 and SPSS for Windows.

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Present address of S. Pirkmajer: Laboratory for Molecular Neurobiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloska 4 SI-1000 Ljubljana, Slovenia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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