Effect of severe short-term malnutrition on diaphragm muscle signal transduction pathways influencing protein turnover

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Lewis, Michael I., Sue C. Bodine, Nader Kamangar, Xuan Xu, Xiaoyu Da, and Mario Fournier. Effect of severe short-term malnutrition on diaphragm muscle signal transduction pathways influencing protein turnover. J Appl Physiol 100: 1799–1806, 2006.—The aim of this study was to evaluate the effect of nutritional deprivation (ND) on signal transduction pathways influencing the translational apparatus in the diaphragm muscle. Male rats were divided into two groups: 1) 20% of usual food intake for 4 days (ND) with water provided at libitum and 2) free-eating control (Ctl). Total protein and RNA were extracted from the diaphragm. Insulin-like growth factor I mRNA was analyzed by RT-PCR. Protein analyses of key cytoplasmic proteins for three signaling pathways deemed important in influencing protein turnover [phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin (mTOR) pathway] were performed by Western blot. Body weight decreased 30% in ND and increased 17% in Ctl animals. Diaphragm mass decreased 29% in ND animals. Muscle insulin-like growth factor I mRNA abundance was reduced 63% in ND animals. ND resulted in a 55% reduction in phosphorylated (Ser21) Akt. Phosphorylation of mammalian target of rapamycin at Ser2448 was reduced 85% in ND animals. Downstream effectors important in translation initiation were also affected by ND. Phosphorylated (Thr495) 70-kDa ribosomal protein S6 kinase was significantly reduced (35%) by ND. ND also resulted in significant dephosphorylation of the translational repressor initiation factor 4E-binding protein 1. Phosphorylation of GSK-3α (Ser21) and GSK-3β (Ser9) was increased 55 and 45%, respectively, with ND. Phosphorylation of ERK1 (Thr202) and ERK2 (Tyr204), p44 and p42, respectively, was reduced 64 and 55%, respectively, with ND. Total protein concentration for all targets in the three pathways was preserved. We conclude that short-term ND altered the phosphorylation states of key proteins of several pathways involved in protein turnover. This forms the framework for future studies aimed at identifying therapeutic targets in the management of short-term nutritionally induced cachexia.

Nutritional deprivation; muscle fiber atrophy; cachexia; mRNA translation

Protein turnover refers to the normal remodeling process, whereby old proteins are continually removed from protein stores (degradation) and replaced with new proteins (synthesis) (40). Disordered protein turnover, if severe, disturbs this delicate balance, resulting in significant loss of protein, particularly in skeletal muscles, the major protein reservoir of the body (40). Protein metabolism is very sensitive to nutritional state, with several studies demonstrating reductions in whole mixed-muscle protein synthesis and enhanced degradation in skeletal muscles (including the diaphragm) after acute nutritional deprivation (ND) (16, 18, 22, 34). The major signals mediating disordered protein turnover with ND are complex and not fully understood. However, reduced levels of insulin-like growth factor I (IGF-I), insulin, amino acids, and enhanced elaboration of corticosteroids are likely important candidates. We previously reported reduced serum levels of IGF-I with acute ND (33) and reduced IGF-I protein levels in the diaphragm muscle of rats subjected to varying degrees of food restriction and body weight loss (32). Furthermore, we reported significant attenuation of diaphragm fiber atrophy in rats after 3 days of complete ND with the concomitant administration of IGF-I by constant infusion (32). In addition, in rats provided with 50% of caloric and protein needs, IGF-I infusion completely prevented atrophy of any diaphragm fiber type (31). Although it would thus appear that IGF-I is a major signal in the context of acute ND, this does not preclude other important influences, such as those mediated by insulin, amino acids, and enhanced endogenous corticosteroid production.

It has recently been reported that the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (Fig. 1) is a major regulator of skeletal muscle hypertrophy and atrophy (5). We hypothesize that additional important signaling pathways are also involved in acute ND-induced muscle atrophy. The aim of this study was therefore to evaluate the effects of short-term malnutrition on three important signal transduction pathways (Fig. 1) involved in protein turnover in skeletal muscle. The signaling pathways are those in which IGF-I (5, 46), insulin (23, 41, 45), amino acids (1, 23, 41, 58), and corticosteroids (37, 51, 52) have direct or indirect effects. Our rationale for comprehensive evaluation of three signal transduction pathways important in protein turnover was to provide a firm basis for future mechanistic studies to improve our understanding of potential molecular targets in the management of disordered protein turnover and diaphragm fiber atrophy with short-term ND.

The studies were performed on the diaphragm muscle, one of the major primary inspiratory muscles. Reduced muscle bulk with ND may have important consequences on the diaphragm with regard to functional force reserve and endurance capacity, which, under conditions of increased demand or
excessive loading, can lead to task (i.e., ventilatory) failure or prolong or prevent weaning from mechanical ventilation. These issues are of major importance in patients with acute and chronic lung disorders (29).

METHODS

Animal Groups and Nutritional Paradigm

Young adult male Sprague-Dawley rats (initial body wt = 167 ± 5 g) were divided into two groups: 1) a free-eating control (Ctl) group (n = 10), with ad libitum access to food and water, and 2) an ND group (n = 9), which was fed 20% of normal food intake (Purina rat chow) all at once, with water provided ad libitum. Specifically, food was withdrawn from Ctl animals 6–8 h before terminal experiments. Terminal experiments were performed in ND animals 18 h after food was provided, which resulted in 24 h of fasting (see Critique of Methods for methodological rationale for both groups). The experimental period lasted 4 days. The animals were individually housed with a 12:12-h dark-light cycle, and ambient temperature was maintained at 22°C. The research protocol was approved by the Burns and Allen Research Institute Animal Care and Use Committee of Cedars-Sinai Medical Center.

IGF-I mRNA Analysis

Total RNA extraction. Total RNA was extracted from 50-mg samples of the left costal diaphragm with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Quality and concentrations of total RNA were determined with a spectrophotometer (SmartSpec 3000, Bio-Rad, Hercules, CA). Samples were stored at –80°C in RNase-free water until analysis.

Oligonucleotides. The primers for IGF-I and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed on the basis of published rat cDNA sequences. Primer sequences for IGF-I (53) were as follows: upstream (5’ to 3’), AAC CTT ACA AAC TCA GCT GCC CCA (bp 685–665); downstream (5’ to 3’), ATG ATG TTC TGG GCT GCC CCA (bp 161–181); upstream (5’ to 3’), ATG ATG TTC TGG GCT GCC CCA (bp 685–665). The expected lengths of the RT-PCR products were 114 bp for IGF-I and 525 bp for GAPDH. GAPDH is a valid housekeeping gene, because it is not affected in catabolic states such as malnutrition and corticosteroid treatment.

Semiquantitative RT-PCR. Oligo(dt) primer (Invitrogen) and Omniscript RT kit (Qiagen, Valencia, CA) were used for RT of 2 μg of total RNA, which yielded 20 μl of cDNA. RT-generated cDNA for IGF-I and GAPDH were amplified using PCR (MJ Research thermal cycler) with the following experimental conditions: initial denaturation at 95°C for 3 min followed by 30 cycles (95°C for 30 s, 60°C for 45 s, and 72°C for 2 min). From each PCR product, 10 μl were loaded on 4% agarose gels and electrophoresed for separation using ethidium bromide for visualization under ultraviolet light. The relative amounts of the PCR products were measured by densitometry (Kodak Electrophoresis Documentation and Analysis System 120) and normalized to levels of GAPDH.

Protein Analyses

Protein extraction. Soluble protein was extracted from 50-mg samples of the right costal diaphragm in cold cell lysis buffer at a 1:10 ratio (Cell Signaling Technologies, Beverly, MA) according to the manufacturer’s protocol. For analysis of mTOR only, 0.4% CHAPS was added to the lysis buffer. Homogenization was performed with a Polytron homogenizer, and homogenates were centrifuged at 14,000 rpm. The supernatant was separated into aliquots in microcentrifuge tubes. Protein concentration was determined using a commercial...
protein assay kit (Bio-Rad) on the basis of the Bradford (6) method and measured with a spectrophotometer (SmartSpec 3000). For mTOR analysis only, the lysate was immunoprecipitated with the primary antibody overnight at 4°C, protein A-agarose beads (Sigma Chemical, St. Louis, MO) were added, and the sample was incubated for another 3 h and washed before the pellet was resuspended in SDS sample buffer and subjected to electrophoresis.

**SDS-PAGE and Western blotting.** Samples were boiled and cooled before they were used for electrophoresis. Protein extracts were loaded on 4–20% linear gradient gels, except initiation factor 4E-binding protein 1 (4E-BP1) and mTOR, for which 10% polyacrylamide gels were used. Proteins were then electrophoretically transferred to nitrocellulose membranes. Blots were incubated with primary antibodies at 4°C overnight, washed, and incubated with an appropriate secondary antibody at room temperature for 1 h. The blots were visualized after development with enhanced chemiluminescence reagents (streptavidin-horseradish peroxidase; Amersham, Piscatway, NJ) according to the manufacturer’s protocol. In a few instances, the blots were reused: they were exposed to stripping buffer (Restore, Pierce Biotechnology, Rockford, IL) and reprobed with a different antibody. Blots were exposed to X-ray film in a cassette, the films were scanned, and identified bands were analyzed by densitometry using a Kodak Analysis System. Western blot data from the ND group were expressed relative to measured mean values from the Ctl group.

**Primary antibodies.** Blots were incubated with antibodies for total Akt, phosphorylated Akt at the COOH terminus (Ser473), total mTOR, phosphorylated mTOR at the regulatory domain (Ser2448), phosphorylated (Thr389) 70-kDa ribosomal protein S6 kinase (p70S6K), the site most closely linked to growth factor stimulation, phosphorylated glycogen synthase kinase (GSK)-3α (Ser21) and GSK-3β (Ser9), ERK1 and ERK2 (p44 and p42, respectively), and phosphorylated ERK1 (Thr202) and ERK2 (Tyr204), obtained from Cell Signaling Technologies. Antibodies for total p70 S6K, total GSK-3β, and total 4E-BP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific 4E-BP1 (PHAS-I) antibody was obtained from Zymed Laboratories (South San Francisco, CA).

**Statistical Analysis**

The distribution of all data was tested for normality, and statistical analysis was performed using a one-way ANOVA (SigmaStat version 2.0, Jandel, Richmond, CA) to compare differences between the independent groups. An α level of 0.05 was used to determine significance. Values are means ± SE.

**RESULTS**

**Body and Muscle Weights**

In ND rats, daily body weights declined progressively, with a significant decrease (by 30%, *P* < 0.01) after 4 days (Fig. 2A). By contrast, the body weight of Ctl animals increased (by 17%, *P* < 0.05; Fig. 2A). Thus, after 4 days, the body weight of ND rats was 47% below expected body weight (*P* < 0.001). In ND rats, diaphragm muscle mass was reduced by 29% compared with Ctl (*P* < 0.0001; Fig. 2B). In ND animals, weights of the tibialis anterior, medial gastrocnemius, and extensor digitorum longus muscles were reduced by 26.4–28.7% compared with Ctl (*P* < 0.001; Fig. 2, B and C), whereas soleus muscle weight was reduced by 19.4% (*P* < 0.05; Fig. 2C).

**Diaphragm Muscle IGF-I mRNA**

A marked reduction in the abundance of IGF-I mRNA was observed in the diaphragm of ND rats after 4 days of severe ND (63% decrease compared with Ctl, *P* < 0.001; Fig. 3).

**Signal Transduction Pathways: Diaphragm Muscle Protein Analysis**

**PI3 kinase-Akt-mTOR pathway.** ND resulted in a significant reduction (55%) in phosphorylated (Ser473) Akt in diaphragm muscle compared with Ctl (*P* < 0.01; Fig. 4A). By contrast, total Akt was not significantly different between the groups.
The effect of ND on phosphorylation of mTOR at Ser2448 was even more striking compared with Ctl (reduced by 85%, P < 0.01; Fig. 4B). Total mTOR was not significantly different between the groups. ND also affected downstream effectors important in mRNA translation. With ND, a 35% reduction in phosphorylated (Thr389) p70S6K was observed in the diaphragm of ND rats compared with Ctl (P < 0.05; Fig. 4C), but no change was noted in total p70S6K between the groups. Furthermore, the phosphorylation state of the translational repressor 4E-BP1 (PHAS-I) was also evaluated (Fig. 4D). With ND, the fully phosphorylated γ isoform of 4E-BP1 was essentially nonexistent (P < 0.0001), the partially phosphorylated β isoform was significantly reduced (56%, P < 0.01), and the unphosphorylated α isoform was significantly increased (>2-fold, P < 0.001).

**PI3 kinase-Akt-GSK-3 pathway.** The levels of phosphorylated (Ser21) GSK-3α were increased (by 55%, P < 0.05; Fig. 5A) and the levels of phosphorylated (Ser9) GSK-3β were similarly significantly increased (by 45%, P < 0.05; Fig. 5B) in the ND diaphragm. By contrast, levels of total GSK-3α and GSK-3β were unchanged by ND (Fig. 5, A and B).

**MAPK-ERK pathway.** Phosphorylated (Thr202) ERK1 (p44) was significantly reduced (by 64%, P < 0.001; Fig. 5C) and phosphorylated (Tyr204) ERK2 (p42) was also significantly decreased (by 45%, P < 0.01; Fig. 5D) in the diaphragm of ND animals. By contrast, levels of total ERK1 and ERK2 (p44 and p42) were not significantly different from Ctl after short-term ND (Fig. 5, C and D).

**DISCUSSION**

Severe short-term ND resulted in significant loss of diaphragm muscle mass. Although the concentration of total protein for signaling intermediates was preserved with ND, altered phosphorylation states of several key proteins of the three signal transduction pathways (important in muscle protein turnover) were comprehensively evaluated.

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Fig. 3. Diaphragm muscle IGF-I mRNA by PCR analysis relative to GAPDH mRNA in Ctl and ND animals. Values are means ± SE. Note 63% decrease in diaphragm IGF-I abundance after 4 days of severe short-term ND compared with Ctl. *P < 0.001 vs. Ctl.

Fig. 4. Total and phosphorylated (P, Ser473) Akt (A), total and phosphorylated (Ser2448) mTOR (B), total and phosphorylated (Thr389) p70S6K (C), and phosphorylation state of 4E-BP1 (PHAS-I, D) in diaphragm of Ctl and ND animals. Values are means ± SE relative to mean Ctl values, except 4E-BP1, which is depicted in mean gray level intensities to allow comparison across isoforms. Phosphorylated Akt, mTOR, and p70S6K were significantly reduced after 4 days of severe short-term ND, while total Akt, mTOR, and p70S6K were preserved with ND. Nonphosphorylated (α) isoform of 4E-BP1 was significantly increased and fully phosphorylated (γ) and partially phosphorylated (β) isoforms of 4E-BP1 were significantly reduced after 4 days of severe short-term ND compared with Ctl. *Significantly different from Ctl.
Critique of Methods

Nutritional paradigm. In developing our nutritional paradigm, our intent was to avoid a postprandial state while still evaluating the muscle signaling milieu that would be most representative of continued muscle fiber growth in free-eating Ctl animals and inhibition of muscle fiber growth/atrophy in ND animals. To achieve this, food was withdrawn from free-eating Ctl rats 6–8 h before terminal experiments. The rationale for this is that >80% of food intake in normal rats occurs during the night (i.e., darkness) (unpublished observations; Refs. 3, 23). Thus our approach simulates a free-eating state while avoiding phosphorylation changes from recent food intake [~1 h postprandial, as commonly reported in the literature (1, 2, 27, 57)] and affords sufficient time for gastric emptying and food absorption. In the ND animals, experiments were performed 18 h after food was provided. Although it is likely that most, if not all, of the food was consumed fairly rapidly, on several occasions, some residual food was observed after a few hours. Thus the ND animals were subjected to ≥14–16 h of fasting. We believe that this condition best represents the muscle signaling milieu responsible for muscle fiber atrophy and an appropriate contrast with the free-eating Ctl animals.

Choice of diaphragm muscle. There is a strong rationale for pursuing studies on the diaphragm, which, as one of the major primary inspiratory muscles constituting the vital air pump of the chest, enables adequate ventilation under resting conditions or with increased demand, as may occur with acute or chronic lung disorders. Clinically, diaphragm dysfunction has a significant impact on the morbidity and/or mortality of patients with acute or chronic lung diseases (29). Our intent was not to generalize our findings to limb muscles, which exhibit very different activation histories (i.e., not phasically recruited throughout life, like the diaphragm, and often quiescent for long periods), loading conditions, fiber type proportions, muscle architecture, and biochemical properties.

Animal model of severe short-term ND: clinical justification. It has recently been emphasized that in the early stage of acute critical illness, there is a low rate of adequate nutrition (13, 19, 38, 44), including the development of malnutrition in pre- (20) and postoperative patients (26) and acute protein/energy malnutrition in children (8, 9). Reasons for this include underprescription and suboptimal delivery of nutrients (13, 38). Furthermore, patients may not tolerate or absorb enteral supplements because of adynamic ileus or other gastrointestinal problems, bowel wall edema and villous atrophy, abdominal bloating, and discomfort (7, 11, 12, 39).

Although previously healthy patients can tolerate short-term ND without any problems, patients with comorbidities and nutritional depletion of cachectic states (e.g., chronic conges-

Fig. 5. Total and phosphorylated (Ser21/9) glycogen synthase kinase (GSK)-3α (A) and GSK-3β (B) and total and phosphorylated (Thr202/Tyr204) ERK1 (p44; C) and ERK2 (p42; D) in diaphragm of Ctl and ND animals. Values are means ± SE relative to mean Ctl values. Note significant increase in phosphorylated GSK-3α and GSK-3β after 4 days of severe short-term ND compared with Ctl and significant reduction in phosphorylated ERK1 (p44) and ERK2 (p42) with ND compared with Ctl. Total protein for GSK-3α, GSK-3β, ERK1, and ERK2 was preserved with ND.
tive heart failure and chronic obstructive pulmonary disease) can suffer a further stepped reduction in respiratory and limb muscle mass that may be difficult to reverse or prevent. A further critical reduction in respiratory muscle mass may significantly prolong attempts to wean patients from mechanical ventilation (25). By contrast, even small improvements in respiratory muscle bulk and strength may have profound influences in the ability to liberate patients from mechanical ventilation (28, 43).

**Signal Transduction Pathways and Downstream Effectors**

Although the phosphorylation states of several key proteins of the three signal transduction pathways evaluated in the diaphragm were altered after 4 days of ND, the total protein concentration for all signaling intermediates was preserved in its entirety during muscle atrophy. We postulate that, as total muscle protein decreased with loss of muscle mass, there was simply a proportional decrease in those signaling protein intermediates. Thus, relative to muscle protein concentration after ND, these signaling proteins would be maintained. However, the proportions of many phosphorylated signaling proteins may have been altered by the impact of the ND paradigm on the energy production system, resulting in a shortage of phosphagen-rich nucleotides, which ultimately impaired the ability of the muscle to maintain the active process of phosphorylation of these signaling intermediates.

**PI3K-Akt-mTOR pathway.** Ligand (e.g., insulin and IGF-I) binding to members of the receptor tyrosine kinase family of growth factor receptors results in a conformational change, which leads to autophosphorylation of multiple tyrosine residues (Fig. 1). For example, PI3K can be activated by direct association with tyrosine-phosphorylated IGF-I receptor or association with insulin receptor substrate 1. Through a number of steps, activated PI3K facilitates the phosphorylation of the serine-threonine kinase Akt (10). Recent data (in muscle cell cultures and intact rat limb muscle) support the hypothesis that enhanced Akt activity results in phosphorylation of a key regulatory domain of mTOR at Ser248 (41, 45, 49). Downstream targets of mTOR include 4E-BP1 and p70S6K. With a low demand for mRNA translation, eukaryotic initiation factor eIF2, which acts to shuttle the initiator methionyl tRNA to the 40S ribosome, thus promoting protein synthesis. Furthermore, mTOR is sensitive to glucose, amino acids, and energy balance, all of which would be expected to be impacted by the severe ND imposed in the present study (15, 41, 50). Recent studies have shown that leucine is the most potent of the branched-chain amino acids in enhancing muscle mRNA translation and that the regulatory role of leucine may be specific for muscle (2).

Our study confirms that the PI3K-Akt-mTOR pathway is significantly affected by severe short-term ND at multiple levels. However, there is paucity of literature on the impact on other signal transduction pathways that can influence muscle protein synthesis. This needs to be further explored, if potential molecular targets, redundancy of pathways, and adaptive changes to severe nutritional insults are to be fully elucidated. **PI3K-Akt-GSK-3 pathway.** GSK-3, a known substrate of Akt, is involved in numerous processes, including glycogen synthesis, protein synthesis, and transcription factor activity (for review, see Ref. 17). Akt phosphorylates and inactivates GSK-3, which diminishes its inhibitory effect on eIF2B. The latter regulates the binding state of eIF2, which acts to shuttle the initiator methionyl tRNA to the 40S ribosome, thus promoting protein synthesis (50). Inhibition of GSK-3β produces hypertrophy of skeletal myotubes in culture (55). In the present study, the significant increments in phosphorylated GSK-3α and GSK-3β were therefore unexpected, inasmuch as our model was characterized by decreased phosphorylation of Akt and prominent muscle fiber atrophy. The unexpected increase in GSK could be explained as follows: 1) GSK-3β could be inactivated by Akt-independent mechanisms. Indeed, phosphorylation by other kinases, such as PKA, PKC-δ, MAPK-1 (also known as p90RSK), and p70S6K (17), has been reported to occur at serine and to inactivate GSK-3. 2) Proteolytic release of amino acids could inhibit GSK-3, possibly via influences on p70S6K (17). 3) The inhibition of GSK-3 may reflect adaptive responses to severe short-term ND. Depletion of glycogen stores is itself a stimulus to promote greater activation of glycogen synthase (presumably through inhibition of GSK-3). One can also speculate that our results regarding GSK-3 reflect adaptations to offset disordered protein turnover and promotion of muscle protein synthesis or other functions of GSK-3 unrelated to glycogen or protein synthesis. It should also be stressed that the increased phosphorylation of GSK-3α and GSK-3β in the present study reflects one point in time at 4 days. Indeed, preliminary data from our laboratory show that at 1 day after acute ND GSK-3β is twice as elevated as at 4 days.
after acute ND (unpublished observations). Thus the level of GSK-3β inactivation appears to decrease with time, reflecting a dynamic state.

**MAPK-ERK pathway.** In the present study, a reduction in phosphorylated ERK1 was observed with ND. In vitro, ERK has been shown to phosphorylate 4E-BP1 independent of any PI3K-Akt signaling (14, 35). In addition, further signaling through ERK-interacting kinase (MNK) may also affect mRNA translation (56). Thus reduced phosphorylated ERK1 may negatively affect translational factors and the translational apparatus important in muscle fiber protein synthesis and maintenance of growth.

**Signal Transduction Pathways and Protein Degradation**

Recently, there has been an increased appreciation of signal transduction events mediating muscle fiber atrophy or hypertrophy that appear to highlight and depend on Akt (24, 47, 48, 54). The importance of these events is that they link and bridge the synthesis and degradation arms of protein turnover. Thus activation of Akt1 can influence signaling pathways, as described above and in Fig. 1, which mediate muscle protein synthesis, while at the same time phosphorylating and inhibiting forkhead box o (Foxo) transcription factors and, thus, blocking their induction of muscle-specific E3 ligases [e.g., MAFbx (atrogin-1) and MuRF1], which are important in ubiquitin-proteasome-mediated proteolysis (4, 21). By contrast, dephosphorylation of Akt1 (as described in our study) would be expected to activate Foxo transcription factors with the subsequent transcription of MAFbx, MuRF1, and other atrophy-related genes to promote muscle protein degradation and wasting. In our recent studies with acute ND, this indeed appears to be the case (30).

**Conclusions and Future Directions**

The present study presents a comprehensive descriptive analysis of aberrations in the muscle protein synthetic pathways, with strong inference for involvement of proteolytic pathways (as suggested above). This forms the framework on which key questions can be addressed (in a cause-and-effect manner) in the genesis of appropriate therapeutic molecular pathways (as suggested above). This forms the framework on which key questions can be addressed (in a cause-and-effect manner) in the genesis of appropriate therapeutic molecular pathways (as suggested above).

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