Environmental Stress Affects the Activity of Metabolic and Growth Factor Signaling Networks and Induces Autophagy Markers in MCF7 Breast Cancer Cells*§

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Phosphoproteomic techniques are contributing to our understanding of how signaling pathways interact and regulate biological processes. This technology is also being used to characterize how signaling networks are remodeled during disease progression and to identify biomarkers of signaling pathway activity and of responses to cancer therapy. A potential caveat in these studies is that phosphorylation is a very dynamic modification that can substantially change during the course of an experiment or the retrieval and processing of cellular samples. Here, we investigated how exposure of cells to ambient conditions modulates phosphorylation and signaling pathway activity in the MCF7 breast cancer cell line. About 1.5% of 3,500 sites measured showed a significant change in phosphorylation extent upon exposure of cells to ambient conditions for 15 min. The effects of this perturbation in modifying phosphorylation patterns did not involve random changes due to stochastic activation of kinases and phosphatases. Instead, exposure of cells to ambient conditions elicited an environmental stress reaction that involved a coordinated response to a metabolic stress situation, which included: (1) the activation of AMPK; (2) the inhibition of PI3K, AKT, and ERK; (3) an increase in markers of protein synthesis inhibition at the level of translation elongation; and (4) an increase in autophagy markers. We also observed that maintaining cells in ice modified but did not completely abolish this metabolic stress response. In summary, exposure of cells to ambient conditions affects the activity of signaling networks previously implicated in metabolic and growth factor signaling. Mass spectrometry data have been deposited to the ProteomeXchange with identifier PXD000472. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.034751, 836–848, 2014.

Phosphorylation is a posttranslational modification involving the addition of phosphate groups to serine, threonine and tyrosine residues on target proteins. This modification, regulated by kinases and phosphatases that phosphorylate and dephosphorylate these amino acids respectively, controls many aspects of protein biochemistry including stability, localization, ability to interact with other molecules and enzymatic activity (1, 2). In addition to playing a pivotal role in regulating most biological processes, alterations in biochemical pathways regulated by protein phosphorylation contribute to the pathophysiology of various diseases including cancer, diabetes and neurodegeneration (2–6).

In recent years the development of MS techniques has allowed the study of protein phosphorylation on an untargeted and global scale. As a consequence, signaling processes can now be studied with unprecedented depth and coverage (7–10). Phosphoproteomics has also been applied to investigate how signaling networks are modulated during disease progression and for the identification of biomarkers that classify patients according to prognosis or treatment response (11–15). A potential caveat in the interpretation of such experiments is that protein phosphorylation is a dynamic modification that can be affected by variables difficult to control including cell confluence, circadian rhythms, shear stress and other types of environmental stresses including exposure to ambient conditions (16–22). Thus, during the course of an experiment variations or delays in sample retrieval and processing can potentially alter the quantitative characteristics of the phosphoproteome (17, 18, 22). Similar problems could in principle occur in a clinical environment where several hours may elapse from patient sample collec-
tion to processing or preservation (16, 17, 23). Delays because of ethical and practical considerations may also affect collection and preservation of post-mortem samples (24, 25). As a consequence, it can in principle be introduced variability and artifacts that may potentially confound the interpretation of data obtained from large-scale as well as targeted phosphoproteomics experiments (16).

To our knowledge, there are no reports that systematically evaluate, in an untargeted manner, how exposure to environmental stress modulates the phosphoproteome of human cells in culture. Here, we used the MCF7 breast cancer cell line to investigate how ambient conditions alter phosphorylation and to evaluate signaling pathways that may be modulated by environmental stress. We found several phosphorylation events that increased or decreased after 15 min exposure of cells to ambient conditions at room temperature (RT)1. We then studied whether these changes in phosphorylation were a random effect due to stochastic inactivation of kinases and phosphatases or whether these were the consequence of actual responses involving specific signaling pathways. Our data indicate that the phosphorylations regulated by environmental conditions at RT are the initial steps of a complex adaptive response to a metabolic stress. Data supporting these conclusions include the observation that ambient conditions at RT activated catabolic pathways regulated by AMPK and GSK3β and inactivated anabolic pathways involving the AKT, ERK and mTOR signaling nodes. We compared the responses to ambient conditions at RT or on ice, we found that maintaining cells on ice induced a different adaptive response rather than an attenuated one. We also found that the adaptation response to ambient conditions at RT triggered a functional biological process that involved the initiation of macroautophagy (hereafter referred as autophagy) and the activation of a pathway known to inhibit protein synthesis at the level of translation elongation. Thus our study also defines experimental conditions that can be used to study the mechanisms involved in the process of autophagy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MCF7 breast cancer cell line was obtained from the ATCC (HTB-22). Cells were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin (each at 100U/ml) at 37 °C in humidified atmosphere at 5% CO2. Cells were maintained at 30–70% confluence. For experiments, cells were seeded in 75 cm2 flask at 35–45% confluence and left overnight. After that, flask containing the cells were left in the incubator (control samples) or exposed to ambient conditions at RT (21 ± 2 °C, RT) or to ambient conditions on ice (temperature = 0 ± 1 °C) for 15 or 120 min (Fig. 1A). Ambient conditions implicate metabolic concentration of CO2 (0.035%). Control cells were lysed within 1 min of being taken from incubators.

1 The abbreviations used are: RT, room temperature; AMPK, AMP activated protein kinase; ERK, Extracellular signal-regulated kinase; eEF2, Eukaryotic elongation factor 2; GSK3β, Glycogen synthase kinase 3β; PIK3C3, Phosphatidylinositol 3-kinase catalytic subunit type 3.
Data Analysis—For phosphopeptide identification, mascot Distiller 2.3.2 was used to smoothen and centroid the MS/MS data and Mascot 2.4 search engine was used to match peaks to peptides in proteins present in the SwissProt Database (SwissProt_2012Oct.fasta) restricted to Human entries (20,309 sequences) (27). The process was automated with Mascot Daemon 2.4.0, mass tolerance was set to 7 ppm and 0.8 millimass units for precursor and fragment ions, respectively. Phosphorylation on Ser, Thr, and Tyr; PyroGlu on N-terminal Gin; and oxidation of Met were allowed in the search as variable modifications and carbamidomethyl Cys as fixed modification. Trypsin was selected as digestion enzyme and 2 miss cleavages were allowed. Sites of modification are reported when they had delta scores >10. Delta scores were calculated as described (28). Otherwise the site of modification was deemed to be ambiguous; in such cases phosphopeptides are reported as the start-end residues within the protein sequence. Results from Mascot searches were deposited into the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [29] with the data set identifier PXD000472 and DOI 10.6019/PXD000472. Phosphopeptides showing a Mascot expectancy of <0.05 (~2% false discovery rate) were placed in a database of peptides quantifiable by MS. Pscal software (26, 30) was used to construct XICs of peptides included in the database across all the samples being compared. Enolase peptides spiked in all samples were used as reference points along chromatograms to align retention times. The XIC windows were 7 ppm and 2 min. Peak height intensity XIC values were normalized to the sum of all values in a sample and average fold change between conditions was calculated. Statistical significance between conditions was considered significant when the Student’s t test produced a p value <0.05. XICs of phosphopeptides showing a significant difference are shown in supplementary Data Set S2.

Western Blots—Cells in the flasks were washed with PBS supplemented with 1 mM Na2VO4 and 1 mM NaF andlyzed in base buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 1 mM EDTA pH 8.0; 1% Triton X-100) supplemented with protease inhibitors (Complete Mini EDTA-free; Roche) and phosphatase inhibitors (1 mM Na3VO4; 1 mM NaF; 1 mM β-glycerol-phosphate; 2.5 mM Na2P2O7; 1 μM okadacinic acid). Cell extracts were incubated on ice for 15 min, scraped from the flask, transfer to 1.5 ml low protein binding eppendorf tubes and centrifuged for 10 min at 13,000 rpm and 4 °C. After Bradford protein quantification, 45 μg of protein were run in 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated in blocking solution (5% milk in TBS-T) for 1 h, primary antibody overnight and secondary antibody for 1 h. Then protein bands were visualized using enhanced chemiluminiscence. Details and concentrations of antibodies used are indicated in Supplemental Table S1.

Immunofluorescence Microscopy—MCF7 cells were seeded in six-well plates on poly-lysine-coated coverslips (1 μg/ml) and cultured for 72 h. The medium was changed 24 h before the start of the experiment and cells in the plates were exposed to RT or Ice conditions during 2 h. Then, cells in the plates were washed with PBS and fixed with ice-cold 100% methanol on ice for 15 min. Samples were blocked with blocking solution (3% BSA in PBS) for 1 h at RT and incubated with primary antibodies overnight at 4 °C. Coverslips were washed three times with PBS and incubated with secondary antibodies for 1h at RT. After three washes in PBS, nuclei were stained using DAPI and coverslips were mounted with Moviol mounting media before imaging. Images were acquired by confocal microscopy (LSM 710 confocal microscope; Carl Zeiss) using 63× oil-immersion objective with an optical slice of 0.8 μm. Puncta were quantified using Meta Morph software (Molecular Devices).

RESULTS

Identification of Metabolic and Growth Factor Networks as Targets of Environmental Stress—To investigate the role of environmental stress in modulating global phosphorylation, the breast cancer cell line MCF7 was exposed to ambient conditions at RT (RT; 21 ± 2 °C and atmospheric concentration of CO2) for 15 min or 2 h, or left in the incubator (Fig. 1A). The 2 h time point was included to investigate whether possible early changes in protein phosphorylation are the initial steps of a complex response or a random effect due to a stochastic inactivation of kinases and phosphatases. The experiment included two completely independent replicates and each experiment was performed twice, giving rise to four biological replicates. Phosphopeptides were quantified by label-free phosphoproteomics using extracted ion chromatograms (XIC) constructed by Pscal (13, 14, 26, 30). In this experimental setup, peptides are quantified in all samples provided they give a positive identification in database search in at least one of the LC-MS/MS runs (30) (Fig. 1A).

We identified and quantified a total of 3,467 phosphopeptides in these experiments, leading to a total of 41,604 quantitative data points. Frequency distributions showed that most peptides were not affected by these perturbations as the average log2-fold changes centered around zero (Fig. 1B and 1C). At arbitrary threshold values of ±0.8-fold change (log2) and p value <0.05, data analysis revealed that the phosphorylation of 53 (28 increased, 25 decreased) and 132 (48 increased, 84 decreased) peptides was affected after exposure of MCF7 cells to ambient conditions at RT for 15 and 120 min, respectively (Fig. 1C; supplementary Data Set S1). Chromatograms used to quantify these phosphopeptides are shown in supplementary Data Set S2.

DAVID pathway analysis (31) revealed, in the set of proteins regulated by exposure to ambient conditions at RT, a significant enrichment of proteins linked to insulin signaling, erb2, EGFR, mTOR and NGF pathways (Table I). The intensities of phosphopeptides on proteins linked to these pathways were mainly decreased as a function of environmental stress (Fig. 2). Interestingly, all these pathways have several kinases in common including AKT, ERK, and mTOR.

Environmental Stress Modulates AKT Signaling—We have previously demonstrated that kinase activity can be estimated by measuring the phosphorylation state of known substrates in an approach named Kinase Substrate Enrichment Analysis (32). After exposure of cells to ambient conditions at RT we observed a reduced phosphorylation of several sites known to be substrates of AKT, including PFKB2 at S466, GSK3β at S9, ACLY at S455, and AKTS1 (PRAS40) at T246, suggesting a reduction in AKT kinase activity, especially after 2 h of perturbation (Fig. 3A). These observations were confirmed by Western blot analysis showing that phosphorylation of AKT substrates GSK3β at S9, TSC2 at T1462, and PRAS40 at T246 were reduced after 2 h exposure to ambient conditions.
at RT (Fig. 3B). Because cells in culture are frequently exposed to ice during sample retrieval and harvesting, we also studied if exposure to ice interferes with the signaling of this kinase. Western blot analysis shows that the phosphorylations of GSK3β/H9252, TSC2, and PRAS40 were also reduced after 2 h exposure to 0 °C (Fig. 3B).

Phosphorylation of S9 on GSK3β inhibits the activity of the kinase (33). This protein kinase phosphorylates residues in the context of SP motifs (34). It was therefore of interest to observe an increased phosphorylation of sites positioned in SP motifs including MAPT at S713/717/721 and IRS-1 at S341 (Fig. 3A), consistent with an activation of GSK3β as a result of its decreased phosphorylation at S9 (35, 36).

AKT is often activated downstream of PI3K and the phosphorylation of AKT at S473 and T308 is frequently used as markers of PI3K activity because they are known to correlate with the activity of the kinase (37). Consistent with a reduction of phosphorylation on AKT substrates, ambient conditions at RT or incubation on ice drastically reduced AKT phosphorylation at T308 or S473 after 2 h incubation. After 15 min exposure to both conditions a clear reduction of AKT phosphorylation at T308 was also observed. However, the phosphorylation at S473 was clearly more reduced when cells were at RT than on ice (Fig. 3B). Thus, these data indicate that PI3K activity is reduced after exposure of cells to environmental stress.
Pathway analysis of phosphopeptides modulated by exposure of cells to RT conditions. Columns show the following information. Category, database mined in the search; term, reference and name of pathway or process returned by the pathway analysis tool; Count, number of proteins undergoing phosphorylations regulated by exposition to RT conditions and classified under the indicated term; %, percentage of proteins (counts) regulated by exposition to RT conditions and matched to the named pathway relative to the total number of proteins undergoing in the dataset; p-Value, significance of the enrichment as determined by the hypergeometric test; Benjamini, adjusted p-Value by the Benjamini-Hochberg procedure.

| Category | Term | Count | %  | p-Value | Benjamini |
|----------|------|-------|----|---------|-----------|
| KEGG     | hsa04012:ErbB signaling pathway | 7    | 7.0| 1.1E-05 | 7.9E-04   |
| KEGG     | hsa04666:Fc gamma R-mediated phagocytosis | 6    | 6.0| 2.4E-04 | 0.0085    |
| KEGG     | hsa04150:mTOR signaling pathway | 5    | 5.0| 2.5E-04 | 0.0060    |
| KEGG     | hsa05213:Endometrial cancer | 5    | 5.0| 2.5E-04 | 0.0060    |
| KEGG     | hsa05221:Acute myeloid leukemia | 5    | 5.0| 3.8E-04 | 0.0068    |
| KEGG     | hsa04722:Neurotrophin signaling pathway | 6    | 6.0| 8.1E-04 | 0.0116    |
| KEGG     | hsa04360:Axon guidance | 6    | 6.0| 9.7E-04 | 0.0116    |
| KEGG     | hsa04910:Insulin signaling pathway | 6    | 6.0| 0.0012  | 0.0122    |
| KEGG     | hsa05210:Colorectal cancer | 5    | 5.0| 0.0015  | 0.0139    |
| KEGG     | hsa04510:Focal adhesion | 6    | 6.0| 0.0067  | 0.0527    |
| REACTOME | REACT_498:Signaling by Insulin receptor | 6    | 5.9| 8.4E-04 | 2.5E-04   |
| REACTOME | REACT_11061:Signalling by NGF | 7    | 7.1| 0.0111  | 0.0162    |
| REACTOME | REACT_9417:Signaling by EGFR | 3    | 3.0| 0.0426  | 0.3530    |

**Fig. 2.** Phosphorylation of proteins in pathways significantly enriched after exposure of cells to RT conditions. Phosphopeptides regulated by exposure of cells to ambient conditions in proteins linked to pathways enriched by these conditions.
Environmental stress modulates mTOR signaling and induces makers of inhibition of translation elongation—mTORC1 (mammalian Target of Rapamycin complex 1), a master regulator of cell growth, protein translation and metabolism (38, 39), signals downstream of the PI3K-AKT signaling pathway. Consistent with the reduction in AKT and PI3K activity, MS phosphoproteomics data showed a reduction in the phosphorylation of known mTORC1 substrates.

**Fig. 3.** Environmental stress inhibits the PI3K-AKT axis and activates GSK3β. MCF7 cells were exposed to ambient conditions at RT or in ice (0 °C) for 15 min or 2 h. Cells were then harvested and processed for phosphoproteomic analysis by MS or by Western blot. A, Phosphopeptides significantly affected by exposure of cells to RT conditions and linked to AKT decrease their abundance (top panel) whereas those linked GSK3β increase (bottom panel). Replicates per condition (N) = 4, F_{15} = average fold-over control (Incu) in log2 after 15 min exposition to RT, F_{2h} = average fold-over control (Incu) in log2 after 2 h exposition to RT, P_{15} = p value of a t test comparing control (Incu) and 15 min exposition to RT, P_{2h} = p value of a t test comparing control (Incu) and 2 h exposition to RT. B, Ambient conditions reduced the phosphorylation of AKT and its substrates.

**Fig. 4.** Environmental stress modulates the phosphorylation of regulators of protein synthesis. Samples were treated and processed as in Fig. 2. A, Effect of RT on phosphopeptides linked to mTOR and S6RPK. Replicates per condition (N) = 4, F_{15} = average fold-over control (Incu) in log2 after 15 min exposition to RT, F_{2h} = average fold-over control (Incu) in log2 after 2 h exposition to RT, P_{15} = p value of a t test comparing control (Incu) and 15 min exposition to RT, P_{2h} = p value of a t test comparing control (Incu) and 2 h exposition to RT. B, Ambient conditions differentially regulate the phosphorylation of regulators of protein synthesis.
including sites in RPS6KB1 (S6K), AKTS1 (PRAS40), LARP1, and FOXK2, after exposure of cells to ambient conditions at RT (Fig. 4A). However, other substrates of mTORC1 including sites at EIF4EBP1 (also known as 4E-BP1) were not affected (Fig. 4A). WB analysis confirmed these selective effects of environmental stress on the mTOR substrates S6K and 4E-BP1 (Fig. 3B). S6K is a kinase whose activity is increased when phosphorylated at T389. No effect was observed by either MS or WB on several phosphorylation sites regulated by this kinase in S6 ribosomal protein (Fig. 4A and 4B). In addition to S6 and 4E-BP1, mTORC1 can regulate translation through other proteins; this includes the phosphorylation and inactivation of eEF2K, a kinase that inhibits the translation elongation factor eEF2 by phosphorylation at T56 (40). MS data showed that ambient conditions at RT induced a decrease in the phosphorylation of eEF2K at S74, a phosphorylation site reported to be sensible to mTOR inhibition (7), suggesting an increase of eEF2K kinase activity by ambient conditions (Fig. 4A). The increased phosphorylation of eEF2 at T56 after exposure of cells to ambient conditions confirmed the activation of eEF2K, thus pointing toward an inhibition of protein synthesis (Fig. 4B). It has also been described that in similar stress conditions protein synthesis can be also inhibited due to the phosphorylation of eIF2α at S51 (41). We were able to detect this phosphorylation when cells were exposed to ambient conditions on ice but not to ambient conditions at RT (Fig. 3B).

Environmental Stress Modulates ERK Signaling—ERK1/2 are other kinases involved in RTK signaling. MS data revealed a decreased phosphorylation of ERK-regulated sites including GAB1 at S551, MYC at S62 and RPS6KB1 at T444/S447 after 2 h exposure to ambient conditions at RT (Fig. 5A). Furthermore, MEK activates ERK1 by direct phosphorylation of the residues T202/Y204. These residues, either on ERK1 (MAPK3) or ERK2 (MAPK1), were dephosphorylated after exposure of cells to ambient conditions at RT. Western blot data confirmed that the effect on ERK1/2 phosphorylation is considerably stronger when the cells were maintained at RT compared with when the cells were maintained on ice, showing no effect after 15 min (Fig. 5B).

In summary, an assessment of the sites dephosphorylated after exposure of cells to ambient conditions indicates that environmental stress inhibited PI3K, AKT, mTOR, and ERK1/2, all of which have known roles in promoting anabolic metabolism.

Environmental Stress Increases Signaling Pathways Involved in Catabolic Metabolism—We also identified several phosphorylation sites that increased in intensity upon exposure of cells to ambient conditions. One of such site was S639 on ULK1, a known substrate of AMPK, a master regulator of cell metabolism that activates catalytic and inhibits anabolic pathways (42, 43). Although several AMPK substrates have been described, it may be assumed that not all of them have been identified. Recently the AMPK consensus motif has been defined using a positional scanning peptide library (42, 44). Motif analysis on our MS phosphoproteomic data showed that the AMPK motif was present in several phosphorylation sites that were highly induced by ambient conditions at RT (Fig. 6A). WB analysis demonstrated that AMPK phosphorylation at T172 was induced whereas phosphorylation at S485 was inhibited upon incubation of cells in ambient conditions (Fig. 6B). Phosphorylation of AMPK T172 is linked to an increase activation of its catalytic activity, whereas phosphorylation at S485 inactivates the kinase (45, 46). In addition, we found an increase in the phosphorylation of ACC1 at S79 and ULK1 at S555 (Fig. 6B); these sites are also both well-known substrates of AMPK (42, 47). Collect-
The data in Fig. 6 indicate that environmental stress induces AMPK activity.

**Environmental Stress Induces Autophagy Markers**—ULK1 is a protein kinase whose activation is implicated in the initiation of autophagy (42). Interestingly, phosphorylation of ULK1 at S555 and S639, found to be increased by our experimental conditions (Fig. 6), is known to promote ULK1 kinase activity (42). In addition, autophagy can be promoted by the activation of AMPK and inhibition of mTORC1 in response to different types of stimuli (48). Thus, we hypothesized that the experimental conditions used in this study may be inducing autophagy. To test this idea, we used immunofluorescence microscopy to measure autophagy in response to incubation of cells in ambient conditions by using two well-known autophagic markers, namely WIPI2 and LC3, that display characteristic puncta staining during the process of autophagosome formation (49). After 2 h exposure of cells to ambient conditions at RT, the number and the size of both WIPI2 and LC3 puncta were dramatically increased (Fig. 7A and 7B). Cells exposed to ice showed an increase in the number of LC3 puncta but not in their size (Fig. 7A and 7B). Furthermore, no increase in the number or size of puncta containing WIPI2 was observed (Fig. 7A and 7B). These data indicate that exposure of cells to ambient conditions at RT triggered the process of autophagy. However, no clear autophagic response was detected after exposure of cells to ambient conditions in ice. Consequently, we investigated whether changes in the phosphorylation of proteins previously implicated in the autophagic process were present in our dataset.

We found an increase in the phosphorylation of proteins known to have roles in autophagy including MATP at T720/S721, BAG3 at S284 and SQSTM1 at S366 in addition to a decrease phosphorylation of MTMR14 at S530 and LARP1 at S766 (Fig. 8) (50–56). These data suggest that environmental stress modulates kinase activities that are involved not only in the signaling pathways that trigger autophagy but also in the actual implementation of the process.

**DISCUSSION**

The impact of environmental stress on cell phosphorylation has not been systematically investigated using untargeted MS-based proteomics. Here we studied the effects of an environmental stress that involves exposing cells to lower temperature (cells maintained at 21 °C, RT) and different CO2 concentration than the one used in cell culture incubators. In other words, we investigated how stress to which cells are often exposed during an experiment affects their phosphoproteome. We found a decrease in the phosphorylation of proteins known to have roles in autophagy including MATP at T720/S721, BAG3 at S284 and SQSTM1 at S366 in addition to a decrease phosphorylation of MTMR14 at S530 and LARP1 at S766 (Fig. 8) (50–56). These data suggest that environmental stress modulates kinase activities that are involved not only in the signaling pathways that trigger autophagy but also in the actual implementation of the process.
are a consequence of a random process due to a stochastic activation or inactivation of kinases and phosphatases or are part of a response to environmental stress that includes particular signaling pathways. Our findings indicate that the changes in phosphorylation observed were not a consequence of a random process but the initial steps of a more complex and coordinated response that was more clearly observed after 2 h of perturbation. This is discussed further below.

Environmental Stress Reduces Anabolic Signaling—Pathway analysis using phosphoproteomics data showed that a significant number of proteins, dephosphorylated as a result of exposure to ambient conditions, were linked to anabolic pathways including insulin, EGF and NGF signaling (Table I and Fig. 2). It should be noted that members of these pathways are common to several RTKs, and therefore, at this level of resolution, our data do not shed light into specific upstream...
receptors. Instead, our data clearly show that growth factor signaling (in broad terms) was decreased as a result of treatment. This is in line with the observation that regulatory sites on kinases driving these pathways, such as those on AKT and ERK1, were dephosphorylated upon exposure to stress (Figs. 3 and 5). This is also consistent with published studies that measured ERK and AKT phosphorylation in a targeted manner as a function of exposure to ambient conditions (22). Dephosphorylation of AKT and ERK was evident after just 15 min of incubation, whereas the effect on their substrates was mainly observed after 2 h (Figs. 3 and 5). This observed delay between the dephosphorylation of these kinases and their substrates may indicate a role for phosphatase activity in the response to these conditions.

Environmental Stress Induce Inhibitory Signals of Protein Synthesis at the Level of Translation Elongation—Most, if not all, types of cellular stresses elicit a global reduction in protein synthesis. This phenomenon may have evolved as a way of saving energy required for undertaking the stress response (57). Translation is a tightly regulated process that is mainly controlled at the levels of initiation and/or elongation. The protein kinase complex mTORC1, which acts downstream of AKT in the insulin signaling pathway (39, 58), is a critical regulator of protein synthesis through its ability to phosphorylate several factors involved in this process including 4E-BP1 and (through S6 kinase) ribosomal S6 (38). However, the reduction in AKT activity (Fig. 3) did not result in a significant decrease in 4E-BP1 or ribosomal S6 phosphorylation (Fig. 4), even though negative regulators of mTORC1, including PRAS40 and TSC2, were dephosphorylated after environmental stress exposure (Fig. 3). These results are consistent with those observed when cells are incubated at 10 °C (41) and indicate that protein synthesis was not affected by the classical mTORC1 pathway that leads to a reduction of 4E-BP1 and S6 phosphorylation.

Translation initiation is also controlled by phosphorylation of the initiation factor eIF2α at S51, a site that, when phosphorylated, inhibits translation initiation by preventing the formation of Met-tRNA-eIF2-GTP ternary complex (57). Cold shock has been reported to decrease protein synthesis by the activation of the stress kinase PERK that phosphorylates eIF2α S51 (41). However, we found that only long exposure times to environmental stress in cold conditions caused the phosphorylation of eIF2α, thus indicating that protein synthesis is not regulated by eIF2α S51 phosphorylation in our system at RT conditions.

Protein synthesis is also regulated at the level of translation elongation. eEF2 is required for the movement of peptidyl-tRNA along the ribosome during translation elongation, a process that is inhibited by phosphorylation of eEF2 T56 (59) and which involves the protein kinase eEF2K (60). We observed a modulation in the phosphorylation of eEF2K by environmental stress (Fig. 4) and an increase in eEF2 phosphorylation (thus inhibition), suggesting that, in our experimental system, environmental stress may inhibit protein synthesis at the level of translation elongation.

Environmental Stress Activates Catabolic Signaling Pathways—We also found that environmental stress induced the phosphorylation of several sites that are substrates of AMPK and modulated phosphorylation sites known to correlate with an activation of the kinase (Fig. 6). AMPK is a crucial energy sensor that regulates cell metabolism by the activation of catabolic and the inhibition of anabolic pathways thus resulting in an increase in ATP levels (43). The decrease of the metabolic rate that occurs at low temperature can decisively contribute to create the situation of low ATP concentration that explains the induction of AMPK activity observed on cells exposed to RT.

Environmental Stress Induces Autophagy Markers—Because exposure of cells to ambient conditions induces the activation and inhibition of specific signaling pathways, we investigated whether these pathways led to the activation of particular biological processes that increase energy availability. It has been shown that AMPK activation and mTORC1 inhibition can trigger autophagy in response to different types of cellular stresses including amino acid or glucose starvation (43, 48, 61). In this process, cytoplasmic contents are degraded to generate nutrients and energy to maintain essential cellular activities (42, 49, 62). The process of autophagy starts with the formation of a membranous structure called phagophore that expands to form a double membrane structure called autophagosome in which cytoplasmic components are sequestered. Eventually autophagosomes fuse with lysosomes to form autolysosomes where the cytoplasmic components are degraded (63). The phagophore formation is controlled by ULK1 and Beclin1-PK3C3 complexes (64, 65). The protein kinases AMPK and ULK1, which is in turn regulated by AMPK and mTORC1 (48), phosphorylate different core and transient components of the PIK3C3-beclin 1 complex, leading to the recruitment and activation of the PIK3C3 at the early phagophores (61, 63). Phosphatidylinositol 3-phosphate (PI3P), the product of the PIK3C3 activity, is necessary for the recruitment of PI3P binding proteins like WIP1 that are essential for the lipidation of LC3 and the formation of the autophagosomes (49). Our data show that environmental stress induces the phosphorylation of ULK1 (Fig. 5) at sites known to correlate with an increase in its kinase activity (42). In addition, there was an increase of the number of puncta positive for WIP1, WIP1/LC3, and LC3 (Fig. 7), consistent with these structures representing phagophores, early autophagosomes and mature autophagosomes, respectively (49). These data demonstrate that autophagy is induced when cells are exposed to environmental stress. When cells were kept on ice the increase of ULK1 phosphorylation was not followed by an increase of WIP1 puncta, thus no clear autophagic response was detected after exposure of cells to ice because LC3 puncta can be formed due to the aggregation of LC3 into inclusion bodies (62, 66).
As with virtually all biological processes, autophagy is regulated by phosphorylation. Recently a study reported the phosphoproteomic analysis of cells treated with resveratrol and spermidine, two agents that induce autophagic responses (67). Because our data points to an induction of autophagy after exposure to environmental stress, we hypothesized that proteins involved in autophagy may be present as phosphoproteins in our dataset. Several phosphorylated sites on such proteins were identified as being modulated by environmental stress (Fig. 8), including those on BAG3, MTMR14, SQSTM1, MATP (also known as Tau), and LARP1, all of which have been implicated in the process of autophagy (50, 52–56, 68). BAG3 is an ATG3 and ATG7 interacting protein involved in the degradation of misfolded and aggregated proteins by autophagy (52, 68). MTMR14 (also known as Jumpy), a negative regulator of autophagy, is a lipid phosphatase that dephosphorylates PI3P, the lipid product of the PIK3C3 complex, and reduces the recruitment of WIPI proteins to the phagophores (53, 55). SQSTM1 (also known as p62) is an adaptor protein that has been implicated in the degradation of polyubiquitinated proteins by autophagy (50). These data imply that environmental stress regulates not only the signaling pathways that trigger autophagy but it also modulates phosphorylation on some of the actual effectors of the process.

MCF7 is a luminal epithelial cell line that is widely used as a model to investigate breast cancer biology (69). This cell line is estrogen receptor positive, ERBB2 negative, contains a mutation on the PIK3CA gene (helical domain) and a homozygous deletion of CDKN2A (69, 70). PI3K signaling is therefore expected to be active in MCF7 cells. AMPK has also been found to regulate many biological processes in MCF7 cells including protein synthesis, cell proliferation and sensitivity to death. AMPK is also known as p38 MAPK, and reduces the recruitment of WIPI proteins to the phagophores (53, 55). SQSTM1 (also known as p62) is an adaptor protein that has been implicated in the degradation of polyubiquitinated proteins by autophagy (50). These data imply that environmental stress regulates not only the signaling pathways that trigger autophagy but it also modulates phosphorylation on some of the actual effectors of the process.

In summary, environmental stress induced during sample retrieval and processing has the potential to modify the phosphoprotein pattern of cells. These modifications, rather than a random change in phosphorylation levels due to stochastic changes in the activity of kinases and phosphatases, represent a coordinated response to a metabolic stress situation. This response involves the inactivation of anabolic pathways regulated by ERK1/2 and the PI3K-AKT-mTOR axis and the activation of catabolic pathways regulated by AMPK. The regulation on these pathways leads to the activation of markers of translation elongation inhibition and to the induction of autophagy.

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