The Impact of Biosampling Procedures on Molecular Data Interpretation*

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The separation between biological and technical variation without extensive use of technical replicates is often challenging, particularly in the context of different forms of protein and peptide modifications. Biosampling procedures in the research laboratory are easier to conduct within a shorter time frame and under controlled conditions as compared with clinical sampling, with the latter often having issues of reproducibility. But is the research laboratory biosampling really less variable? Biosampling introduces within minutes rapid tissue-specific changes in the cellular microenvironment, thus inducing a range of different pathways associated with cell survival. Biosampling involves hypoxia and, depending on the circumstances, hypothermia, circumstances for which there are evolutionarily conserved defense strategies in the range of species and also are relevant for the range of biomedical conditions. It remains unclear to what extent such adaptive processes are reflected in different biosampling procedures or how important they are for the definition of sample quality. Lately, an increasing number of comparative studies on different biosampling approaches, post-mortem effects and pre-sampling biological state, have investigated such immediate early biosampling effects. Commonalities between biosampling effects and a range of ischemia/reperfusion- and hypometabolism/anoxia-associated biological phenomena indicate that even small variations in post-sampling time intervals are likely to introduce a set of nonrandom and tissue-specific effects of experimental importance (both in vivo and in vitro). This review integrates the information provided by these comparative studies and discusses how an adaptive biological perspective in biosampling procedures may be relevant for sample quality issues. *Molecular & Cellular Proteomics* 12: 10.1074/mcp.R112.024869, 1489–1501, 2013.

The understanding of how specific observations at the molecular level relate to properties of the entire living organism is one of the greatest challenges in biomedical research. Observations can be influenced significantly by both the methodolo-

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utes difference in sampling time can, for instance, have pro-
dependent on enzymatic processes, singling out the pro-
mechanisms underlying tissue-protective processes.

Such adaptive processes depend both on the adaptive systems inherent in the organism in question and the exact conditions of the pre-sampling state of the organism. In animal research, there are major differences in euthanasia protocols both with regard to methodology (such as asphyxia, terminal anesthesia, or decapitation in the case of smaller rodents such as mice) and the time required for their performance. Yet, there is little discussion as to what extent adaptive biological reactions occur during different forms of biosampling and how they might influence the interpretability of results and/or extrapolation between experiments. Biological evolution has resulted in a range of different life strategies (Table I) determining the ability of the organisms to adapt and tolerate hypoxia and anoxia and hypothermia and hypometabolism (the latter two being connected with tolerance toward anoxia). Compared with temperature-conforming organisms, endothermic (warm-blooded) organisms have a low tolerance toward anoxia and hypometabolism/hypothermia. This is exemplified by the insufficient adaptation that underlies ischemia-reperfusion-induced damage (3). An animal can be resuscitated after a short period of global ischemia, rapidly adapting to the reduced oxygen levels, and yet become unable to handle the sudden reintroduction of oxygen. Intriguingly, such damage can sometimes be ameliorated by therapeutic hypothermia, a result that is likely to depend on more than just the consequences of a reduced metabolism. A reduction of core temperature in rats correlates with a drop of ~5%/°C in the cerebral metabolic rate of oxygen (4), meaning that the observed protection in animals, and possibly humans, at 4–5°C reduction, occurs at a metabolic reduction of only 20–25%. The neuroprotective effects invoked by anesthesia against ischemia/reperfusion damage (5, 6) also indicate that cells initially try to adapt to the hypometabolism and/or hypothermia associated with biosampling. The mechanisms underlying such physiological changes need to have an extremely short response time and are therefore heavily dependent on enzymatic processes, singling out the proteome as being particularly sensitive to the choice of both sampling methodology and type of tissue (7–12). A few minutes difference in sampling time can, for instance, have profound effects on the peptidome (here defined as all proteins <10 kDa) (13, 14), yet it easily remains within the acceptable time frame for sampling in most biomedical research. In contrast, some peptidomics biomarker signatures are dependent on protease activity induced during post-sampling handling rather than pre-sampling levels (15). Choosing whether to heat or freeze a sample can result in ~30% difference (8) in results at the proteome level (here defined as all proteins >10 kDa) (Fig. 1, A and B). This brings into question our definition of sample quality, the nature of these biosampling differences, and their implications for research methodology and data interpretation, especially when investigating the molecular mechanisms underlying tissue-protective processes.

### DEFINING SAMPLE QUALITY

Here, sample quality is referred to as an attribute of biosampling applied to both living and dead organisms. The highest sample quality is achieved when sampling is performed in a manner optimal for experimental reproducibility and the representativeness of the organism’s living state. The dynamic nature of the proteome and its role in adaptive responses makes it difficult to exactly gauge the representativeness of a sample to its pre-sampling state, at least when investigating regulatory networks involved at the post-translational level in adaptive responses.

When looking at in vivo metazoan biology, achieving the highest sample quality presents numerous challenges as multicellular organisms by definition are composed of multiple, semi-autonomous living parts. From the biological perspective, death can be seen both as a multilayered chain-of-events spanning different organizational layers and as a more specific event when an organism is no longer able to adapt to its surroundings at the individual or cellular level. In the case of individual death, most cells continue living as far as their semi-autonomous nature allows them to cope with the drastically changing microenvironmental conditions. Consequently, the relation between the death of an organism and sample quality can be perceived on several levels (Fig. 1C) as follows. 1) “Individual death” indicates the individual (metazoan) has lost its ability to adapt to its macroenvironment, but most cells remain alive. 2) “Cellular death” indicates cells have lost the ability to adapt to their microenvironments, but their molecules retain some biochemical function due to remaining

### TABLE I

| Survival strategy | Organisms | Anoxia sensitivity |
|-------------------|-----------|--------------------|
| Ectothermal strategy, temperature-conforming | Fish, amphibians, reptiles | Usually extensively tolerant to anoxia, hypometabolism, and hypothermia (88, 89) |
| Endothermal strategy, some mammals use temporary hypometabolic states: daily metabolic rate depression (torpor) or seasonal (aestivation, hibernation) | Mammals and birds, tachymetabolic organisms | Weak tolerance to anoxia |

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cellular integrity and/or proximity. 3) "Chemical activity" indicates molecules, such as enzymes, are inactive, but remain susceptible to nonenzymatic changes, such as oxidation, during sample handling and storage. 4) "Chemical inactivity" indicates a biosample storage-dependent level when all chemical reactions are on hold.

Tissues and organs, not being semi-autonomous adaptive systems with homeostatic properties, are not included in this hierarchy as they cannot be considered to be alive in the individual or cellular sense but rather are bioactive thanks to their component cells. They constitute the closest environment of the remaining living cells. It is generally agreed that for a sample to be in a state as close as possible to its pre-collection, in vivo condition, biosampling methods must attenuate and/or stop all molecular changes (enzymatic or chemical), either by inactivating an entire specimen, for example by snap-freezing, or by disrupting sample integrity, for instance using homogenization and/or strongly denaturizing inactivation buffers.

**Bioreaction Termination and Bioreaction Termination Intervals**—The literature describes biosampling in a relatively flexible manner. For clarity, we will use "bioreaction termination" (BT)¹ to describe general biosample inactivation and "bioreaction termination interval" (BTI).

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¹ The abbreviations used are: BT, bioreaction termination; BTI, bioreaction termination interval; CFI, chemical fixation inactivation; CHS, conductive heat stabilization; MCI, mechano-chemical inactivation; PTM, post-translational modification; SFI, snap-freezing inactivation; SFI + MCI, snap-freezing followed by mechano-chemical inactivation; SNO, S-nitrosylation; AMPK, AMP-activated protein kinase; NMDA-R, NMDA receptor.
action termination interval" (BTI) as the time period from individual death or sample extraction from a life-supporting environment to the actual sample inactivation. The term "bioreaction" does not require the organism to be alive (i.e. neither at the individual nor cellular level) but rather reflects all biochemical reactions that continue to occur in the biosample. As a concept, it therefore encompasses all reactions that continue to occur in extracted tissue, in *in vitro* derived cell samples, or in nominally cell-free bodily fluids such as urine. The post-mortem interval as well as the post-excision phase/delay time (16) are therefore BTIs applied to tissue extraction biosampling.

Depending on the choice of sampling approach, there are temporary and terminal forms of BTs. Temporary BT methods only transiently inactivate bioreactions and are dependent on a subsequent terminal BT step. Terminal BT methods applied immediately after sample extraction are, while having some drawbacks (see Table II) (17), arguably superior to temporary BT methods when it comes to minimizing the BTI of biosampling and providing a more realistic representation of the molecular state in the living organism. The main BTs used include the following: 1) snap-freezing inactivation (SFI, a temporary BT); 2) lyophilization inactivation (or freeze-drying, a temporal BT); 3) mechano-chemical inactivation (MCI, a terminal BT); 4) chemical fixation inactivation (CFI, a terminal BT), and 5) heat stabilization (a terminal BT); 4) chemical fixation inactivation (CFI, a terminal BT), and 5) heat stabilization (a terminal BT). The first method, snap-freezing, is the most commonly used and requires dry ice or liquid nitrogen as a cooling medium. Lyophilization is a related cooling approach, providing a temporary method that enables the storing of viable cells (18). Mechano-chemical inactivation uses some mechanical means (for instance mechanical shears, a glass-Teflon homogenizer, or bead homogenization) to disrupt sample structure (i.e. sample homogenization), often in the presence of inactivating sample buffer. It is often performed subsequent to snap-freezing in most laboratory protocols ("SFI+MCI").

The retained bioactivity in snap-frozen biosamples allows a wider range of downstream analysis than heat-inactivated samples, but it may provide a false sense about the molecular activity present in the pre-extraction sample. For instance, preparing snap-frozen tissues for immunohistochemistry via microtome followed by thaw-mounting on slides allows the reinstatement of biochemical reactions such as mitochondrial cytochrome c oxidase activity (19).

Chemical fixation inactivation can involve both cross-linking (such as formalin) and noncross-linking (for instance the PAXgene tissue fixation system (20)) fixation reagents. The cross-linking nature of the former is a limiter for subsequent molecular studies in general and proteomic studies in particular. Some forms of chemical fixation inactivation, such as formalin fixation, extend the BTI by several hours allowing a range of biochemical reactions to occur (21). Rapid heating by conductive heat stabilization (CHS) has been demonstrated to be more efficient than the SFI+MCI approach in minimizing BTI-associated biological changes (8, 19, 22), and subsequent heat stabilization of snap-frozen tissue negates the majority of normal SFI+MCI-induced changes demonstrating that the thawing of samples invokes a range of enzyme activity (8, 23). Focused microwave irradiation is also used as a rapid heat stabilization source but is currently limited to small animal brain tissue applications. All heat-conductive inactivation methods are detrimental for any subsequent morphology studies.

**Biosampling, Post-mortem Effects**—The BTI (post-mortem interval) after individual death significantly influences results at the molecular level. Human post-mortem studies and forensic science studies tend to have extended BTIs, from 20 to 30 min and longer, whereas the BTI in experimental animal research is usually less than 10 min for smaller animals. With animals, the BTI is usually minimized as much as possible, although materials and methods sections tend to vary considerably in the details of this stage. Post-mortem sampling occurs after cervical dislocation, blunt trauma to the head in mice, or by euthanasia by injection (which result in shorter BTIs) or inhalation (resulting in longer BTIs). The effects on molecular signaling from the combination of the more specific influence from ante-mortem conditions and immediate post-excision phase are poorly characterized. In the case of inhalation euthanasia in animals, usually spanning several minutes, the choice of terminal anesthesia may introduce a neuroprotective hypometabolic state (see below), whereas asphyxiation causes a gradual anoxia (as compared with decapitation-induced anoxia). Molecular research involving neural tissue from anesthetized animals (BTI <80–90s) is more likely to represent a pre-depolarization state (5) as compared with the state in nonanesthetized animals (BTI >50s) but, depending on variations in animal handling and sample extraction, may result in biosamples representing a mix of both states. Animal models with reduced brain metabolism, for instance some Alzheimer models, are therefore likely to exhibit different neural tissue sensitivities to BTIs compared with nonhypometabolic controls. The phosphorylation pattern of a central neurodegeneration protein, Tau, is known to be sensitive to both anesthesia and hypometabolism (24, 25).

**Biosampling, Biopsies, and *in Vitro*—With the exception of surgical biopsies, biosampling from living organisms is generally limited to relatively easily accessible parts of the body. Human surgical biopsies are common but have a larger variation in the time span (often from ~30 min up to several hours) from procurement to storage, with the latter usually in liquid nitrogen. Clinical histological analysis of tissue biopsies has to contend with a number of variables (16) besides issues of extended BTIs and the slowness of the BT methodology itself. Espina et al. (2) have subdivided the BTI for the post-excision phase/post-excision delay time of surgically extracted tissues into wounding/excision followed by pro-survival and apoptosis/cell death, reflecting the phosphoprotein alterations in different pathways.
TABLE II

Overview of biotermination methods

| Bioreaction termination | Snap-frozen<sup>a</sup> | Lyophilization<sup>b</sup> | Inhibitors,<sup>d</sup> protease, kinase, phosphatase | Strong denaturizing sample buffer | Formalin fixation, cross-linking fixative | PAXgene tissue system,<sup>e</sup> precipitation fixative | Heat stabilization, conductive,<sup>f</sup> microwave<sup>g</sup> |
|-------------------------|-------------------------|---------------------------|----------------------------------------------------|---------------------------------|----------------------------------------------|---------------------------------------------------|-----------------------------------------------|
| Speed of inhibition      | Temporary               | Temporary                 | Temporary                                          | Terminal/terminal<sup>h</sup> | Terminal                                      | Terminal                                          | Terminal                                       |
| Subdissection at RT      | Rapid                   | Rapid                     | Slow<sup>a</sup>                                   | Slow<sup>a</sup>               | Slow                                         | Slow                                             | Slow                                          |
| Price                    | Low                     | High                      | Moderate                                           | Low                             | Low                                           | Moderate                                         | High                                          |
| Toxic                    | Low                     | Low                       | High                                               | High                            | High                                          | High                                             | Low                                           |

Data dependent on bioreaction termination from frozen state (can be combined with lyophilization, strong denaturizing sample buffers, or heat stabilization).<sup>a</sup>

<sup>b</sup> See Ref. 18.

<sup>c</sup> Strong denaturizing buffers or inhibitors can also be used without the help of a mechano-chemical method.<sup>d</sup>

<sup>d</sup> See Ref. 90.

<sup>e</sup> See Ref. 20.

<sup>f</sup> See Ref. 19.

<sup>g</sup> See Ref. 43.

<sup>h</sup> Depending on the strength of the buffer, it can be more or less permanent, and there is a risk of enzymatic activity when diluting the buffers.

<sup>i</sup> See Ref. 91.

<sup>j</sup> See Ref. 92.
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Biopsies of excreted body fluids such as tears, saliva, breast milk, and urine are often used for biomarker studies and require the inhibition of any potential biochemical activity and chemical modifications. Blood is of particular interest in the context of biopsies and biomarkers. It is classified as a connective tissue and, in this sense, biosampling involves extraction of a biosample with its own particular microenvironmental cell-supporting conditions. As an interface and/or conduit between tissues and organs, blood is both a transport medium and an absorbent system for unspecific tissue diffusion. It consists of endogenous and exogenous components, the former being those cells and molecules associated with its transport functionality. Heart-specific isoforms of troponin are biomarker examples of exogenous blood components as they appear 3–5 h post-infarction (26). Their abundance is dependent on the severity of the infarction. Examples of commonly used biomarkers requiring short BTIs are glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide, glucagon, and ghrelin (27). In addition, the complement activation and coagulation pathways present in blood introduce a specific set of protease-associated bioreactions, influencing protein degradation patterns during the BTI (15).

Another common type of biosampling is from in vitro systems such as cell and tissue cultures. The experimental conditions of the cultures determine the extent of sudden changes experienced by the cells. The BTI from the initial disruption of the life-supporting culture conditions to final sample handling varies a lot between protocols, many being relatively short (a few minutes). However, cells in culture at the same time are far more closely connected to a stressful environment than cells in tissue biopsies, which are enclosed in the organizational structural remainder of the pre-sampling tissue, and therefore are likely to be more susceptible to even small perturbations. Extensive proteomic differences have been observed in a comparative BT study on yeast cells (28). Differences in culturing conditions and BTIs and BT methodology, such as the extent of time washing the cells, breaking up adherence by trypsination, biomechanical pressure effects, and limited access to oxygen during centrifugation, all combine to influence the degree to which the final sample represents the pre-sampling in vitro state.

BIOLOGY OF BIOREACTION TERMINATION INTERVALS

What are the biochemical consequences of choosing a biosampling approach and to what extent do they matter? Fig. 1D depicts a flow chart of the biosampling process in post- and ante-mortem animals. The combinatorial use of SFI and other subsequent BT methods (for instance SFI+MCI, see Fig. 1E) adds the additional BTI time period (T2) that reflects the thawing period of the temporary frozen biosample before terminal inactivation. If samples are rapidly terminally inactivated while the tissue is still deeply frozen, T2 becomes noninfluential.

The thawing and/or sample homogenization process (8) demonstrates relatively large differences between heat stabilization (CHS) and immediate MCI or SFI+MCI even when T2 is minimized and strong inhibitors are included. If thawing and/or weak homogenizations are conducted over a longer time period (T2 is several minutes), cells are more likely to retain their integrity longer, thereby determining the context for any chemical reactions, adaptive (full integrity) or reactive (reduced integrity). After the initial stage (or if MCI is performed immediately and efficiently), any subsequent chemical activity is dependent on four factors as follows: the concentration of molecules; the properties of the buffer; the initial proximity of the molecules at the point of cellular lysis, and their affinity toward each other. Immediate but weak homogenization after biosampling and without subsequent SFI is likely to induce similar effects.

From this perspective, “weaker” detergent-based sample buffers are more likely to retain more active molecular complexes (high affinity and close proximity) than strong buffers, even in the presence of different inhibitors. Adaptive processes during the overall pre-snap-freezing or MCI stage are likely to determine the configuration of molecular complexes associated with acute stress adaptation and are dependent on two main factors, inherent survival strategies (Table I) and associated tissue properties. Anoxia tolerance depends on the basal metabolic rate and body temperature together with the specific requirements of individual tissues. For example, mammalian cardiac tissues possess oxygen reserves in the form of myoglobin, an attribute that helps to delay the initial post-mortem BTI response (29).

Another pro-survival strategy for cells is to maintain energy reserves via autophagy and protein catabolism (30). One of the main cellular effects of anoxia is a gradual drop in mitochondrial ATP production, this being the main O2 consumer, followed by a shift toward anaerobic energy production and hypometabolism. A drop in cellular ATP production induces a massive reduction in DNA/RNA and protein synthesis and redistribution of the remaining ATP to supportive functions (31–35). There is a strong case to be made that membrane permeability in combination with metabolic rate are important factors in explaining why certain tissues are tolerant or sensitive to hypoxia and hypothermia (36). The hypothesis that induction of hypometabolism in combination with the reduction of the ATP required for cellular membrane potential purposes, “channel arrest,” are the primary tools for cells to become hypoxia-/anoxia-tolerant, is supported by studies on membrane channel and receptor functionality in mollusks, turtles, and hibernating mammals (37–39). In the absence of a normal de novo synthesis, cells have only limited means to adapt to their novel situation. One would therefore expect them to use easily produced or already present biomolecules for this purpose. Post-transcriptional regulation with noncoding RNAs is one such example and has been observed in the context of hibernation (40). Post-translational modification of
proteins (PTMs) is another example (see below). Although there are differences between species, there very likely remains some similarity in underlying stress-adaptive mechanisms. Proteins identified in the hypoxic/anoxic tissue of ecotothermal organisms (41, 42) overlap with those identified in BT methodology studies (10, 12, 14, 17), for example glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase, triose-phosphate isomerase, dihydropyrimidinase-related protein 3, and voltage-dependent anion-selective channels.

Post-translational Modifications

One of the main adaptive tools available to the cell is the regulation of proteins through PTMs, which are also known to be particularly sensitive to the choice of BT and BTIs (2, 7, 14, 19, 43). As long as the decreasing supply of ATP and other secondary energy sources such as creatinine phosphate allows it, enzymes will use phosphorylation and other reversible PTMs to increase flux through the glycolytic pathway and shift mitochondrial catabolism and anabolic lipid and protein pathways from an active to a suppressed state. The dependence on ATP for PTMs creates two levels of BTI-associated alterations, an immediate ATP-dependent stage and an ATP-independent stage. Kinases require ATP, whereas phosphatases involve hydrolysis, meaning that any immediate BTI-adaptive changes involving both kinases and phosphatases will be further modified by the ATP-independent reactivity of phosphatases. This is seen as an initial increase in overall protein phosphorylation followed by a reduction (2).

Snap-freezing methods are associated with increased phosphatase activity, an effect that is only mildly attenuated by the presence of phosphatase inhibitors (19). Immediate SFI+MCI on hippocampal and cortical tissues (with a reported BTI of ~30 s) initially causes a higher level of phosphorylation for JNK1/2, CaMKIIα and CaMKIIβ, GSK3β, Akt, and RSK1 (7), indicative of adaptive or reactive cellular activity, whereas biosamples left for 30 min before SFI+MCI show a general reduction in phosphorylation levels. The activation of certain pathways during the initial BTI is counterbalanced by the inactivation of other pathways through directed phosphatase activity. Neural phosphorylation levels of ERK threonine and tyrosine (Thr-202 and Tyr-204) in mouse striatum and cortex become reduced within 1 min and exhibit extensive reduction within 3 min (7, 14). Overall, there are major differences between SFI+MCI and heat stabilization at the neuroproteomic level (Figs. 1B and 2A) (8, 10, 12). In less anoxia-sensitive human uterine biopsies (2), a number of signaling pathways (including PI3K-Akt and Ras-ERK) have increased phosphorylation levels during the first 30 min of BTI before eventually decreasing. Interestingly, although many signaling proteins become dephosphorylated after this initial peak, most proteins reach a subsequent steady state at 60–80% of the initial phosphorylation level for at least an additional hour, indicating a balancing effect of phosphatase activity (2).

Redox Modifications—Oxygen is the basis for two adaptive PTM classes, hydroxylation and S-nitrosylation (SNO). Oxygen pressure determines the hydroxylation level of the hypoxia-responsive protein, hypoxia-induced factor (HIF1α), with anoxia leading to minimal hydroxylation. HIF1α regulates the shift from mitochondrial to glycolysis energy production (44), an activity that is further stabilized by SNO. SNO occurs on cysteine thiol side chains and requires nitrous oxide (NO) provided by NOS enzymes (inducible NOS, endothelial NOS, and neuronal NOS) and the reversion of the NO metabolite nitrite. Endothelial NOS is increasingly phosphorylated at Akt/PKB and AMPK activating target sites during the first 2 h of BTI in human uterine leiomyoma biopsies (16). The reversion to NO allows prolonged SNO activity, a process catalyzed by deoxygenated hemoglobin, deoxygenated myoglobin, neuroglobin, xanthine oxidoreductase, carbonic anhydrase (45–48), and mitochondrial electron transport enzymes such as cytochrome c oxidase (49–51). Because of the presence of endothelial NOS, vascular tissues are particularly prone to SNO activity in response to acute hypoxia (52, 53). Inhalation euthanasia in animal models may therefore give a different redox-associated proteomic profile than more rapid euthanasia methods.

Sumoylation—Protein SUMOylation is a reversible and tissue-protective process induced by hypoxia, is sensitive to different types of cellular stress (54–57), and may therefore also be a feature of BTIs. This modification is related to the ubiquitination pathway but requires fewer enzymes for its functionality and includes HIF1α among its substrates (58). In mouse brain cortex, samples with a BTI of ~30 s contain more SUMO-3 compared with samples with a BTI of 30 min but not more SUMO-1 and -2 monomers (7). The exact mechanism behind SUMOylation-derived tissue protection is unclear but is likely to be part of the cellular PTM-based shift to low ATP levels using a hypometabolic state as it causes repression of gene transcription in hibernating or hypothermic mammalian systems through the modification of transcription factors (57, 59).

Acetylation—Acetylation, like phosphorylation, is an evolutionarily conserved reversible PTM system that influences a large proportion of the proteome and is closely linked to metabolism and mRNA expression regulation (60). Although more extensive studies on BTI effects on acetylation modifications are lacking, anoxia (5 h–20 h) in warm-blooded animals is associated with altered H3K9ac and Histone deacetylase (HDAC) levels (61) and HDAC inhibitors ameliorate the effects of ischemia/reperfusion (62, 63). In general, prolonged BTIs (~5–50 h) cause altered neuroprotein acetylation levels (64) and more specifically influence H3K9ac and H3K27ac (65). This contrasts with reports on another chromatin marker, cytosine methylation, that re-
mains stable during prolonged BTIs (66). BTI acetylation effects are also implied by the enrichment of BT-sensitive and acetylation-regulated proteins in studies of four tissues (Fig. 2B). Among the BT-sensitive proteins, GAPDH is especially interesting in this context as it is transported into the nucleus after SNO modification where it causes the activation of lysine acetyltransferases (CBP/p300) and inhibition of deacetylases (HDAC2 and SIRT1) (67, 68), thereby linking redox signaling to acetylation regulation (Fig. 2A). This involvement of protein acetylation in initial BTI adaptation may have consequences for mechanistic epigenetics studies, particularly in neuroepigenetics. As histone lysine acetylation is closely linked to same residue methylation, any such changes are also likely to involve histone methylation changes.

Akt/PKB and AMPK Signaling

The serine/threonine kinase Akt (also known as PKB) is likely one of the main regulators in initial BTI-induced cellular hypoxia/anoxia adaptation. It belongs to the pro-survival insulin/growth hormone and Ca$^{2+}$-sensitive PI3K-Akt pathway, a SFI-MCI-sensitive set of proteins (7) involved in mammalian hibernation processes (69), neuroprotection against ischemia, and hypothermia-mediated cardiac protection after cardiac arrest (70, 71). Akt regulates the protein synthesis/metabolism controlling mTORC complexes (mTORC1 and mTORC2) (72, 73) together with disulfide isomerase (ERP57) (8–10), another BT-sensitive protein. Both Akt and ERP57 are targets of SNO (74, 75) with ERP57 also being a redox-sensitive chaperone in the endoplasmatic reticulum (75) and transporter of extracel-

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**Fig. 2. Overview of immediate early BTI-sensitive pathways.** A, pathway schemes of signaling proteins (AMPK, Akt, and MAPK pathways), some of their downstream substrates (including mTOR complexes), and their sensitivity to BT. Dark purple nodes are proteins identified by two-dimensional electrophoresis methods as sensitive to BT choice (snap-frozen and heat-stabilized). Light purple nodes are proteins showing increased phosphorylation in snap-frozen samples as compared with heat-stabilized samples. Green nodes are proteins found to have unaltered or reduced levels of phosphorylation in snap-frozen samples relative to heat-stabilized samples. Circular gray nodes are phosphatases (PTEN, PP1, and PP2A). The sno-prefix reflects some proteins with known S-nitrosylation modifications (HDAC2 and SIRT1, ERP57, Akt, and HIF1α). B, enrichment analysis of UniProt annotations in proteins identified to be specifically sensitive to BT choice in brain, heart, liver, and pancreatic tissue (DAVID-based calculation (86); *, adjusted p < 0.05). Based on nonredundant protein identities, an enrichment of acetylation modification is common for all tissues. C, balance between neuronal NMDA-R-associated neuroprotection and neurodegradation. Physiological NMDA-R signaling involves neuroprotective pathways such as the PI3K-Akt pathway. Increased NMDA-R-associated calcium uptake induces increased calcium protease activity, reactive oxygen species (ROS) and nitric oxide (NO) generation, and alters mitochondrial functionality (80, 87). D, BTI duration effects on cAMP-response element-binding protein (CREB)-Ser-133 phosphorylation (CREB-Ser(P)) in formalin-fixed (CFI) mouse cortex tissue. Regular formalin fixation causes extensive dephosphorylation of cAMP-response element-binding protein due to slow tissue penetration, an effect that is prevented by initial heat stabilization (CHS+iCFI).
lular NO into cells. Altered PI3K-Akt pathway activity (76) has been linked to anesthesia, a condition associated with hypometabolism, which delays neural membrane depolarization by approximately ~30 s from 50 to 80 s after death by decapitation (5, 77). Tau, a substrate to Akt-associated GSK3β, is sensitive to anesthetic agents and anesthesia-induced hypothermia (24, 25, 78, 79). The neuronal PI3K-Akt pathway is activated by physiological NMDA-R activity, a glutamate receptor whose increased activation is also of major importance for ischemia-associated cell damage (Fig. 2C). This points to a close linkage between neuronal neurodegradation and neuroprotection/adaptive processes (80). Damaging levels of NMDA-R-associated Ca$$^{2+}$$ cause increased Ca$$^{2+}$$ protease activity, which is likely to be one of the reasons behind the BTI-induced degradome profile in neural biosamples (see below). Ca$$^{2+}$$ proteases such as calpain (81) are rapidly elevated during ischemia and are also able to inhibit PI3K/Akt pathway activity. The AMPK is another mTORC complex-regulating protein that is sensitive to the initial choice of biosampling methodology, in particular when followed by a freeze-thaw process (23).

**BTI-associated Degradation**

Sample quality is often defined by the extent of “sample degradation” or “reduced sample integrity” and linked to the amount of degradation fragments (the degradome in the case of proteins) present in a biosample. The degradome is composed of enzyme-dependent (proteases) and -independent (hydrolysis) process products, the former being a form of PTM. Not surprisingly, proteolytic degradation is often seen as a reactive rather than an adaptive process. Sample handling generally involves proteases getting access to cellular compartments normally kept protected, thus providing new proteolytic substrates. Even a very short BTI (<3 min) or using SFI+MCI is associated with a clear increase in peptides (8, 14). This image is not as clear with larger proteins (>10 kDa). Proteomic profiles derived from SFI+MCI neural tissue and in vitro yeast biosamples demonstrate an increase in low molecular weight proteins and a reduction in high molecular weight proteins (some of the former being fragments of the latter), a trend absent in SFI-handled biosamples from heart, liver, and pancreatic tissue (8, 10, 28). The neural tissue degradation profile is possibly a reflection of its high sensitivity to ischemia and/or the dissipation of ion gradients and subsequent rise in Ca$$^{2+}$$ concentration (causing an elevation of Ca$$^{2+}$$ protease activity) (77, 82). The absence of sufficient ATP reduces the re-uptake of glutamate in the synaptic clefts and inhibits the active export of intracellular calcium, thereby enabling activation of calcium-sensitive proteases such as calpain (80). The increased level of degradation products present in snap-frozen yeast samples is therefore interesting as it implies that at least some in vitro samples are as sensitive as neural tissues to BT choice and BTI extent. What is less understood is to what level, if any, the components of the initial BTI degradome possess bioactive properties (i.e. proteolytic fragments with independent biological properties), how cleavage relates to other forms of PTMs (for instance creating novel modification sites), and/or what such degradomes may mean for biomarker analysis.

Peptides from the BTI-sensitive Akt pathway-associated CRMP2 protein have been reported to be neuroprotective against NMDA-R-associated excitotoxicity, possibly through the reduction of NMDA-R-mediated Ca$$^{2+}$$ influx (83). Presence of proteolytic fragment bioactivity is also hinted at in BT-comparative peptidomics of the pancreas (8). Specific peptide regions within the insulin 1 C-peptide (“founder peptide”) are known to stimulate Na$$^+$$/K$$^+$$-ATPase (84) and are over-represented in its default (CHS-derived) pancreatic degradation profile (Fig. 3A). The major cluster of C-peptide fragments follows a C-terminal gap (VARQ), a sequence reported to have the full activity of the C-peptide (84) in stimulating Na$$^+$$/K$$^+$$-ATPase activity. It represents a set of peptides that all contain a region with moderately effective Na$$^+$$/K$$^+$$-ATPase activity. A smaller, lighter cluster also contains this somewhat less efficient Na$$^+$$/K$$^+$$-ATPase-stimulating fragment, indicating the presence of an additional pool of potentially C-peptide-derived active peptides. In certain cases, the sample handling introduces a specific sequence of protease activity (15, 85). A first tier of protease activity in human serum involves complement activation pathways and coagulation producing a set of founder peptides (for instance bradykinin, fibrinopeptide A, and complement C3F). A second tier of protease activity from blood exogenous proteases is believed to subsequently target these founder peptides (15). These exogenous proteases are likely health state-specific proteins diffused from different tissues, and post-sampling protease activity can occur very rapidly. For instance, addition of the synthetic complement C3F founder peptide to fresh plasma leads, within seconds, to removal of the C-terminal arginine and, within 10–15 min, to truncation of the N-terminal (Fig. 3B). Tumors are believed to contribute to a range of blood exogenous proteases, creating subtle cancer-specific degradome/biomarker signatures (85). This also illustrates one additional subtlety inherent in the notion of sample quality. In contrast with the aims of most functional studies, molecular biomarkers are not required to be directly involved in the core processes underlying any particular disease as long as they establish a robust association to that disease state. In the case of cancer serum degradomes, biosample quality is defined by its representativeness of the protease activity state (i.e. the degradome) in patients suffering from cancer rather than the absolute levels of those proteases (Fig. 3B).

**CONCLUDING REMARKS**

Great effort is put into standardizing molecular biology methodologies and their associated data handling and into
reducing technical variation as much as possible. Although reducing technical variation is clearly worthwhile, the biological adaptive processes inherent in biosampling have received much less attention. This is for instance reflected in commonly used terminology (focusing on “sample degradation” and “reduced sample integrity”), which implies an initial default state that subsequently loses its integrity rather than a set of adaptive processes. Research articles on anoxia and stress-associated phenomena are often very specific in mentioning the exact extent of oxygen deprivation but are more vague on the exact circumstances surrounding BTI and BT. In the same vein, although phosphatase and protease inhibitors are a relatively common component in sample buffers to stop dephosphorylation and proteolysis, attenuation of additional phosphorylation via kinase inhibitors receives much less attention. Overall, the molecular biology literature places relatively little focus on the distinctions between different sampling methodologies, time intervals before sample inactivation, or even the different pre-sampling states of the organisms involved (such as anesthetized or conscious animals).

Espina et al. (2, 16) reached a similar conclusion in their tissue biopsy studies on post-excision delay-induced protein modification effects. They noted that excised biosamples can no longer be viewed as nonreactive or unchanging after procurement (2, 16). The present overview of immediate early changes after sampling and comparisons between biosampling methodologies indicates that such issues are relevant even before the time frame investigated by Espina et al. (2, 16) (>10 min post-excision phase and upward). All biosamples, with the possible exception of in vivo microwave- inactivated brain samples, possess a BTI that influences the representativeness of the sample. The relevance of the BTI extent and the BT methods employed, and the notion of representativeness in sample quality, is always dependent on the purpose for the biosampling. As the example with the cancer serum degradomes (Fig. 3B) demonstrates, representativeness in biomarker studies is not always directly correlated with the shortest possible BTI but with the power for classification. A somewhat longer BTI actually increases the representativeness in this context (15).

Mechanistic studies tend to be more interested in the absolute representativeness of the pre-sampling state. This, paradoxically, is likely to increase variability in immediate early BTI time frames. Taking the pre-sampling state, organism/tissue properties, and acute adaptive reactions into account means that as individual researchers attempt to reduce the BTI, the more likely it is that small differences in time, especially with neural tissue, within and between experiments (and laboratories) will introduce greater variability in certain classes of biomolecules and make it more difficult to distinguish biological from technical variation. With the exception of peptides and peptide fragments, such immediate early BTI effects are unlikely to influence the measurement of the total levels of most proteins but rather involve their post-translational modifications. In conclusion, there is a need for a more differentiated approach to biosampling in experimental biology and biomarker studies. Increased attention to such issues is of
value for the interpretation of molecular biology data and biomarkers.

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REFERENCES

1. Poste, G. (2011) Bring on the biomarkers. Nature 469, 156–157

2. Espina, V., Edmiston, K. H., Heiby, M., Pierobon, M., Sciro, M., Merritt, B., Banks, S., Deng, J., VanMeter, A. J., Geho, D. H., Pastore, L., Sennesh, J., Petricoin, E. F., 3rd, and Liotta, L. A. (2008) A portrait of tissue phosphoprotein stability in the clinical tissue procurement process. Mol. Cell. Proteomics 7, 1998–2018

3. Han, F., Da, T., Riobo, N. A., and Becker, L. B. (2008) Early mitochondrial dysfunction in electron transfer activity and reactive oxygen species generation after cardiac arrest. Crit. Care Med. 36, S447–S453

4. Hågberg, M., Harp, J., Nilsson, L., and Siesjö, B. K. (1975) The effect of induced hypothermia upon oxygen consumption in the rat brain. J. Neurochem. 24, 311–316

5. van Rijn, C. M., Krijnen, H., Menting-Hermeling, S., and Coenen, A. M. (2011) Decapitation in rats: latency to unconsciousness and the “wave of death”. PLoS ONE 6, e16514

6. Schiffrillii, D., Grasso, G., Conti, A., and Fodale, V. (2010) Anaesthetic-related neuroprotection: intravenous or inhalational agents? CNS Drugs 24, 893–907

7. Ahmed, M. M., and Gardiner, K. J. (2011) Preserving protein profiles in tissue samples: Differing outcomes with and without heat stabilization. J. Neurosci. Methods 196, 99–106

8. Scholz, B., Sköld, K., Kultima, K., Fernandez, C., Waldemarson, S., Savitski, M. M., Söderquist, M., Borén, M., Stella, R., Andrén, P., Zubarev, R., and James, P. (2011) Impact of temperature-dependent sampling procedures in proteomics and peptidomics—a characterization of the liver and pancreas post-mortem degradome. Mol. Cell. Proteomics 10, M900229MCP200

9. Kennedy, S. A., Scaife, C., Dunn, M. J., Wood, A. E., and Watson, R. W. (2011) Benefits of heat treatment to the protease packed neutrophil for proteome analysis: halting protein degradation. Proteomics 11, 2560–2564

10. Robinson, A. A., Westbrook, J. A., English, J. A., Boreén, M., and Dunn, M. J. (2009) Assessing the use of thermal preservation to treat the intact proteomes of post-mortem heart and brain tissue. Proteomics 9, 4433–4444

11. Che, F.-Y., Lim, J., Pan, H., Biswas, R., and Fricker, L. D. (2005) Quantitative neuromolecular proteomics of microwave-injured mouse brain and pituitary. Mol. Cell. Proteomics 4, 1391–1405

12. Snejkal, G. B., Rivas-Morelo, C., Chang, J.-H., Freeman, E., Trachtenberg, A. J., Lazarev, A., Ivanov, A. R., and Kuo, W. P. (2011) Thermal stabilization of tissues and the preservation of protein phosphorylation states for two-dimensional gel electrophoresis. Electrophoresis, 32, 2206–2215

13. Svensson, M., Sköld, K., Svenningsson, P., and Andren, P. E. (2003) Peptidomics-based discovery of novel neuropeptides. J. Proteome Res. 2, 213–219

14. Sköld, K., Svensson, M., Normann, M., Sjögren, B., Svenningsson, P., and Andren, P. E. (2007) The significance of biochemical and molecular sample integrity in brain proteomics and peptidomics: stathmin 2-20 and peptides as sample quality indicators. Proteomics 7, 4445–4456

15. Villanueva, J., Shaffer, D. R., Philip, J., Chaparro, C. A., Erdjument-bromage, H., Olshen, A. B., Fleisher, M., Lilja, H., Brogi, E., Boyd, J., Sanchez-carbayo, M., Holland, E. C., Cordon-cardo, C., Scher, H. I., and Tempst, P. (2006) Differential exoproteome activities confer tumor-specific serum peptide patterns. J. Clin. Invest. 116, 271–284

16. Espina, V., Mueller, C., Edmiston, K., Sciro, M., Petricoin, E. F., and Liotta, L. A. (2009) Tissue is alive: New technologies are needed to address the problems of protein biomarker pre-analytical variability. Proteomics Clin. Appl. 3, 874–882

17. Kultima, K., Sköld, K., and Borén, M. (2011) Biomarkers of disease and post-mortem changes—Heat stabilization, a necessary tool for measurement of protein regulation. J. Proteomics 75, 149–159

18. Natan, D., Nagler, A., and Arav, A. (2009) Freeze-drying of mononuclear cells derived from umbilical cord blood followed by colony formation. PLoS ONE 4, e5240

19. Svensson, M., Borén, M., Sköld, K., Falth, M., Sjögren, B., Andersson, M., Svenningsson, P., and Andren, P. E. (2009) Heat stabilization of the tissue proteome: a new technology for improved proteomics. J. Proteome Res. 8, 974–981

20. Ergin, B., Meding, R., Langer, R., Kap, M., Viertler, C., Schott, C., Furch, E., Riegm, P., Zatloukal, K., Walsh, A., and Becker, K.-F. (2010) Proteomic analysis of PAXgene-fixed tissues. J. Proteome Res. 9, 5188–5196

21. Borén, M. (2011) Methods in Molecular Biology, 1st Ed, Springer, Clifton, N.J., 1000

22. Kokkat, T. J., McGarvey, D., Lovecchio, L. C., and LiVoisi, V. A. (2011) Effect of thaw temperatures in reducing enzyme activity in human thyroid tissues. Biopreservation and Biobanking 9, 349–354

23. Scharf, M. T., Mackiewicz, M., Naidoo, N., O’Callaghan, J. P., and Pack, A. I. (2008) AMP-activated protein kinase phosphorylation in brain is dependent on method of killing and tissue preparation. J. Neurochem. 105, 833–841

24. Planel, E., Richter, K. E., Nolan, C. E., Finley, J. E., Liu, L., Wen, Y., Krishnamurthy, P., Herman, M., Wang, L., Schacht, J. B., Nelson, R. B., Lau, L.-F., and Duff, K. E. (2007) Anesthesia leads to tau hyperphosphorylation through inhibition of phosphorylase activity by hypothermia. J. Neurosci. 27, 3090–3097

25. Cummins, B., Auckland, M. L., and Cummins, P. (1987) Cardiac-specific troponin-I radioimmunoassay in the diagnosis of acute myocardial infarction. Am. Heart J. 113, 1333–1344

26. Theodorsson-Norheim, E., Hemsén, A., Brodin, E., and Lundberg, J. M. (1987) Sample handling techniques when analyzing regulatory peptides. Life Sci. 41, 845–848

27. Grassl, J., Westbrook, J. A., Robinson, A., Borén, M., Dunn, M. J., and Clyne, R. K. (2009) Preserving the yeast proteome from sample degradation. Proteomics 9, 4616–4626

28. Hendgen-Cotta, U. B., Filög, U., Keln, M., and Rassaf, T. (2010) Unmasking the human face of myoglobin in health and disease. J. Exp. Biol. 213, 2734–2740

29. Kuma, A., and Mizushima, N. (2010) Physiological role of autophagy as an intracellular recycling system: with an emphasis on nutrient metabolism. Semin. Cell Dev. Biol. 21, 683–690

30. Chang, W. W., Huang, L., Shen, M., Webster, C., Burlingame, A. L., and Roberts, J. K. (2000) Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. Plant Physiol. 122, 295–318

31. Smith, R. W., Houlihan, D. F., Nilsson, G. E., and Brechin, J. G. (1996) Tissue-specific changes in protein synthesis rates in vivo during anoxia in crucian carp. Am. J. Physiol. 271, R897–R904

32. Larade, K., and Storey, K. B. (2002) Reversible suppression of protein synthesis in concert with polysome disaggregation during anoxia exposure in Littorina littorea. Mol. Cell. Biochem. 232, 121–127

33. Storey, K. B., and Storey, J. M. (2007) Tribute to P. L. Lutz: putting life on “pause”–molecular regulation of hypometabolism. J. Exp. Biol. 210, 1700–1714

34. Frerichs, K. U., Smith, C. B., Brenner, M., DeGracia, D. J., Krause, G. S., Marrone, L., Dever, T. E., and Hallenbeck, J. M. (1998) Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. Proc. Natl. Acad. Sci. USA 95, 14511–14516

35. Hochachka, P. W. (1986) Defense strategies against hypoxia and hypothermia. Science 231, 234–241

36. MacDonald, J. A., and Storey, K. B. (1999) Regulation of ground squirrel NaK-ATPase activity by reversible phosphorylation during hibernation. Biochem. Biophys. Res. Commun. 254, 424–429
Impact of Biosampling Procedures on Molecular Data

38. Rammanan, C. J., and Storey, K. B. (2006) Suppression of Na+/K+-ATPase activity during estivation in the land snail Otala lactea. J. Exp. Biol. 209, 677–688

39. Bickler, P. E., and Buck, L. T. (2007) Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu. Rev. Physiol. 69, 145–170

40. Morin, P. J., Dubuc, A., and Storey, K. B. (2008) Differential expression of microRNA species in organs of hibernating ground squirrels: a role in translational suppression during torpor. Biochim. Biophys. Acta 1779, 628–633

41. Bosworth, C. A., 4th, Chou, C.-W., Cole, R. B., and Rees, B. B. (2005) Mitochondrial cytochrome oxidase produces nitric oxide under inflammatory conditions. FEBS Lett. 579, 193–197

42. Smith, R. W., Cash, P., Eliefsen, S., and Nilsson, G. E. (2009) Proteomic characterization of a murine carp brain during exposure to anoxia. Proteomics 9, 2217–2229

43. O’Callaghan, J. P., and Sriram, K. (2004) Suppression of Na+K+-ATPase activity during estivation in the land snail Otala lactea. J. Exp. Biol. 209, 677–688

44. Kim, J. W., Tchernyshyov, I., Semenza, G. L., and Dang, C. V. (2006) Akt-mediated activation of the nutrient-sensitive raptor-mTOR pathway and complex. J. Comp. Physiol. B 177, 175–195

45. Koshy, V. M., Ramírez-Rangel, I., Bracho-Valdez, I., Vázquez-Macías, A., Carretero-Ortega, J., Reyes-Cruz, G., and Vázquez-Prado, J. (2011) Regulation of mTORC1 complex assembly and signaling by GRp58/ERp57. Cell Reports 1, 264–275

46. Shiva, S., Huang, Z., Grubina, R., Sun, J., Ringwood, L. A., MacArthur, M. T., Fago, A. (2009) Generation of nitric oxide from nitrite by carbonic anhydrase in eukaryotes. J. Physiol. Heart Circ. Physiol. 297, H2068–H2074

47. Miller, T. M., Stevens, C. R., Benjamin, N., Esenhalter, R., Harrison, R., and Blake, D. R. (1998) Xanthine oxidoreductase catalyses the reduction of nitrites and nitrate to nitric oxide under hypoxic conditions. FEBS Lett. 427, 225–229

48. Aamand, R., Dalsgaard, T., Jensen, F. B., Simonsen, U., Roepstorff, A., and Fago, A. (2009) Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. Am. J. Physiol. Heart Circ. Physiol. 297, H1590–H1597

49. Noht, H., Staniek, K., Sobohian, B., Bahrami, S., Redli, H., and Kozlov, A. V. (2000) Mitochondria recycle nitric oxide back to the bioregulator nitric monoxide in the human heart. FEBS Lett. 470, 913–921

50. Castello, P. R., David, P. S., McClure, T., Crook, Z., and Poyton, R. O. (2006) Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. Cell Metab. 3, 277–287

51. Kozlov, A. V., Staniek, K., and Noht, H. (1999) Nitrite reductase activity is a novel function of mammalian mitochondria. FEBS Lett. 454, 127–130

52. Chen, S., Huang, B., Liu, Y. C., Shyu, K. G., Lin, P. Y., and Wang, D. L. (2008) Acute hypoxia enhances proteins’ S-nitrosylation in endothelial cells. Biochem. Biophys. Res. Commun. 377, 1274–1278

53. Chen, S.-C., Liu, Y.-C., Shyu, K.-G., and Wang, D. L. (2008) Acute hypoxia to endothelial cells induces activating transcription factor 3 (ATF3) expression that is mediated via nitric oxide. Atherosclerosis 201, 281–288

54. Comerford, K. M., Leonard, M. O., Karhausen, J., Carey, R., Colgan, S. P., and Taylor, C. T. (2003) Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. Proc. Natl. Acad. Sci. U.S.A. 100, 986–991

55. Manza, L. L., Codreanu, S. G., Stamler, S. L., Smith, D. L., Wells, K. S., Roberts, R. L., and Liebler, D. C. (2004) Global shifts in protein sumoylation in response to electrophile and oxidative stress. Chem. Res. Toxicol. 17, 1706–1715

56. Agbor, T. A., Cheong, A., Comerford, K. M., Schoiz, C. C., Bruning, U., Clarke, A., Cummins, E. P., Cagney, G., and Taylor, C. T. (2011) Small ubiquitin-related modifier (SUMO)-1 promotes glycolysis in hypoxia. J. Biol. Chem. 286, 4718–4726

57. Lee, Y. J., Miyake, S., Wakita, H., McMullen, D. C., Azuma, Y., Auh, S., and Hallenbeck, J. M. (2007) Protein SUMOylation is massively increased in hibernation torpor and is critical for the cytoprotection provided by ischemic preconditioning and hypothermia in SH-SY5Y cells. J. Cereb. Blood Flow Metab. 27, 950–962
78. Whittington, R. A., Virág, L., Marcouiller, F., Papon, M.-A., El Khoury, N. B., Julien, C., Morin, F., Emala, C. W., and Planel, E. (2011) Propofol directly increases tau phosphorylation. PLoS ONE 6, e16648
79. Tan, W., Cao, X., Wang, J., Lv, H., Wu, B., and Ma, H. (2010) Tau hyperphosphorylation is associated with memory impairment after exposure to 1.5% isoflurane without temperature maintenance in rats. Eur. J. Anaesthesiol. 27, 835–841
80. Hardingham, G. E. (2009) Coupling of the NMDA receptor to neuroprotective and neurodestructive events. Biochem. Soc. Trans. 37, 1147–1160
81. Beltran, L., Chaussade, C., Vanhaesebroeck, B., and Cutillas, P. R. (2011) Calpain interacts with class IA phosphoinositide 3-kinases regulating their stability and signaling activity. Proc. Natl. Acad. Sci. U.S.A. 108, 16217–16222
82. Lipton, P. (1999) Ischemic cell death in brain neurons. Physiol. Rev. 79, 1431–1568
83. Brittain, J. M., Chen, L., Wilson, S. M., Brustovetsky, T., Gao, X., Ashpole, N. M., Molosh, A. I., You, H., Hudmon, A., Shekhar, A., White, F. A., Zamponi, G. W., Brustovetsky, N., Chen, J., and Khanna, R. (2011) Neuroprotection against traumatic brain injury by a peptide derived from the collapsin response mediator protein 2 (CRMP2). J. Biol. Chem. 286, 37778–37792
84. Ohtomo, Y., Bergman, T., Johansson, B. L., Jörnvall, H., and Wahren, J. (1998) Differential effects of proinsulin C-peptide fragments on Na¹,K¹-ATPase activity of renal tubule segments. Diabetologia 41, 287–291
85. van den Broek, I., Sparidans, R. W., Schelliens, J. H., and Beijnen, J. H. (2010) Specific investigation of sample handling effects on protease activities and absolute serum concentrations of various putative peptidome cancer biomarkers. Clin. Proteomics 6, 115–127
86. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57
87. Yoon, J. S., Mughal, M. R., and Mattson, M. P. (2011) Energy restriction negates NMDA receptor antagonist efficacy in ischemic stroke. Neurobiol. Med. 13, 175–178
88. Jackson, D. C. (2000) Living without oxygen: lessons from the freshwater turtle. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 125, 299–315
89. Ullsch, G. (1989) Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. Biol. Rev. 64, 435–516
90. Mueller, C., Edmiston, K. H., Carpenter, C., Gaffney, E., Ryan, C., Ward, R., White, S., Memeo, L., Colarossi, C., Petricoin, E. F., 3rd, Liotta, L. A., and Espina, V. (2011) One-step preservation of phosphoproteins and tissue morphology at room temperature for diagnostic and research specimens. PLoS ONE 6, e23780
91. Galli, C., and Racagni, G. (1982) Use of microwave techniques to inactivate brain enzymes rapidly. Methods Enzymol. 86, 635–642
92. Delaney, S. M., and Geiger, J. D. (1996) Brain regional levels of adenosine and adenosine nucleotides in rats killed by high-energy focused microwave irradiation. J. Neurosci. Methods 64, 151–156