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Phosphoproteomics taken to heart

Alicia Lundby[1,2,*] and Jesper V Olsen[1,*]

[1]Novo Nordisk Foundation Center for Protein Research; Department for Proteomics; Faculty of Health and Medical Sciences; University of Copenhagen; Copenhagen, Denmark; [2]The Danish National Research Foundation Centre for Cardiac Arrhythmia; Copenhagen, Denmark

The last decade has witnessed a tremendous advance in mass spectrometric instrumentation, sample preparation, and computational proteomics developments that now make it possible to analyze proteins[1] or post-translational modifications (PTMs) of proteins[2] on a global scale and compare their relative abundance between different cell states. As the proteomics technology rapidly advances, it is becoming increasingly popular in cell biology, where it has especially proven a powerful tool to characterize cellular responses by analyzing global protein phosphorylation changes in a stimulus- and time-resolved manner.[3] Protein phosphorylation is tightly regulated in the cell by the action of kinases and phosphatases, and it is involved in regulating essentially all cellular processes, where site-specific phosphorylation events often function as molecular switches that change or fine-tune the action of target proteins, either by altering their enzymatic activity or by affecting interaction partners or subcellular localization.

In our group, we have, in the last few years, focused our efforts on developing robust and reproducible methods to analyze PTMs, such as phosphorylation and acetylation, on proteins extracted from tissue samples.[4,5] This is an important step for the impact of proteomics in biology, as it opens new avenues for investigating cell signaling networks in vivo. There is no doubt that quantitative phosphoproteomics has revolutionized the investigations of cell signaling networks in a global and unbiased manner, but the investigations have so far largely been limited to cell culture models. However, for many physiological processes, it is not sufficient to investigate the responses elicited by a given stimulus in cell culture, as immortalized cell lines lack many tissue-specific proteins. One such example is proteins involved in excitation–contraction coupling of the heart. In a recent study, for the first time, we investigated cardiac β-adrenergic signaling on a global scale by analyzing the phosphorylation site changes of proteins extracted from murine hearts that were treated with β-blockers and activators,[6] thus performing quantitative phosphoproteomics in vivo.

Adrenaline stimulates β-adrenergic receptors (βAR) as an essential component of the "fight-or-flight" response in human physiology, resulting in increased cardiac output mediated by increased contractile force and heart rate. Activation of the βARs initiates protein phosphorylation-dependent signaling cascades that increase myocardial contractility and relaxation rate. β-blockers that inhibit βARs are widely used in the clinic to prevent cardiac arrhythmias and treat hypertension, but knowledge of their downstream molecular targets remains limited. Therefore, delineating the cardiac signaling pathways regulated by phosphorylation as a result of βAR stimulation bears important etiological and therapeutic implications in diseases such as hypertension and heart failure. In our published work,[6] we treated a control group of mice with specific βAR inhibitors and another group of mice with βAR activators (**Fig. 1**). To delineate the downstream effectors of βAR activation, we excised the hearts of the mice and subjected them to phosphoproteomics investigation. We identified more than 600 phosphorylation sites on 300 proteins that are significantly regulated by the stimulus. Our data set covers all previously described regulatory phosphoproteins in this response, but, importantly, it expands our knowledge of βAR-regulated phosphorylation sites from tens to hundreds. Our data supports the notion of important roles of the PKA and CamKIID kinases in the response, but we further provide evidence for involvement of the AMPK and AKT kinases. We also show that 6 ion channels and transporters that are important regulators of cardiac excitability have increased phosphorylation levels. For the Kv7.1 voltage-gated potassium channel, which controls cardiac repolarization, we demonstrated that βAR induced phosphorylation of S92 occurs on channels residing at the plasma membrane, and that phosphorylation increases the current conduction of the channel. In a physiological context, this finding provides mechanistic insight into how a faster repolarization of cardiomyocytes is supported by the Kv7.1 channel upon βAR stimulation, which is required for a faster heart rate. By providing molecular details of which proteins are regulated by site-specific phosphorylation upon β-adrenergic stimulation, we furthermore present a framework to identify therapeutic targets for pharmacological intervention as an alternative to the β-blockers currently prescribed.

Our work represents a state-of-the-art elucidation of an in vivo signaling network important in human physiology.[7] As deregulated signaling is a hallmark of several severe diseases, such as cancer, diabetes, and neuropsychiatric disorders, we foresee that our approach will lay the foundation for future in vivo investigations of signaling pathways. We believe the in vivo

*Correspondence to: Alicia Lundby; Email: alicia.lundby@cpr.ku.dk; Jesper V Olsen; Email: jesper.olsen@cpr.ku.dk
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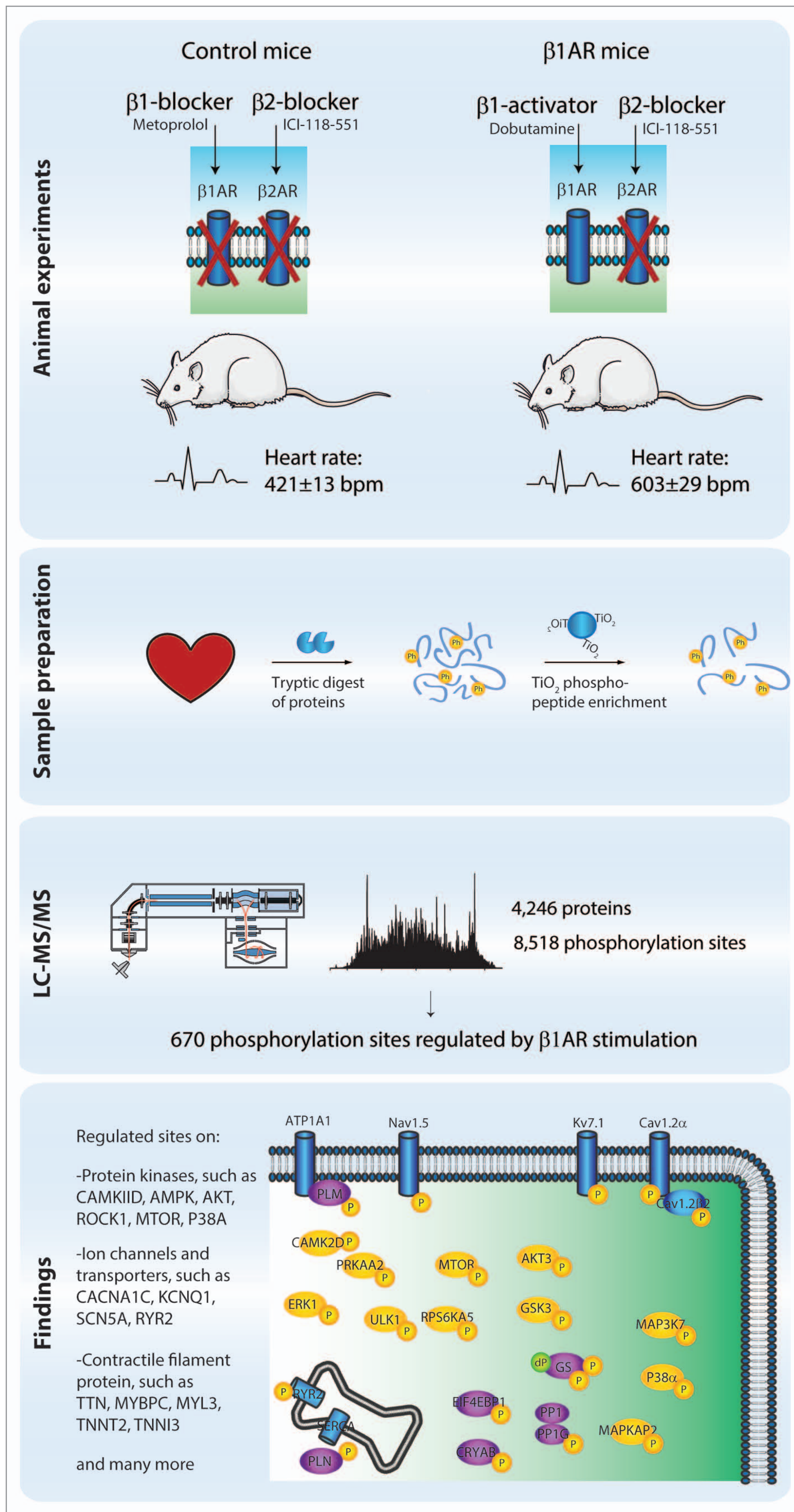

Figure 1. Experimental procedure: 2 groups of mice were treated with either blockers against β1AR and β2AR (control animals) or with a blocker against β2AR and an activator of β1AR (β1AR animals). β1AR animals were stimulated for 10 min. Proteins were extracted from the cardiac tissue and digested with trypsin followed by phosphopeptide enrichment by titanium-dioxide chromatography. The resulting samples were analyzed by high-resolution orbitrap tandem mass spectrometry to a depth of more than 4000 phosphorylated proteins containing more than 8500 phosphorylation sites. We quantified the phosphoproteins and phosphorylation sites between the 2 groups of mice. Six hundred and seventy phosphorylation sites were statistically significantly regulated in the β1AR animals compared with the control animals. The results highlighted several unexpected protein kinases with a sequence-specific preference of R-X-X-pS/T, as well as pinpointing the exact amino acid regulated by phosphorylation on key proteins involved in cardiac excitation–contraction coupling (figure adapted from Lundby et al.).[6]

applications of quantitative phosphoproteomics will grow tremendously in the coming years, where it has the potential to contribute significant new findings to physiological sciences.

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