Exploring the impact of hypoxia on gonadal steroidogenic cells: new insights from proteomics and spheroid development in Leydig cells

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Abstract

The modulatory role of reduced oxygen (O_2) tension (hypoxia) is an essential part of physiological steroidogenic processes. Hypoxia inducible factor (HIF) 1α, a master regulator in biological responses to hypoxia, has been implicated as a direct transcriptional regulator of Steroidogenic Acute Regulatory (STAR)-mediated steroidogenesis. Studies have shown that the degree of hypoxia, duration of exposure to reduced O_2 tension, as well as the tissue/cell type and the stability of HIF1α, impact steroidogenesis to varying degrees. Here, we aimed to identify the largescale changes that occur in the proteome of Leydig cells mediated by cAMP, HIF1α and O_2 levels using a quantitative LC-MS/MS approach. Two Leydig cell lines (MLTC1 and MA10) were used, treated with (db)cAMP or without (control) for 6h in serum-free medium under different O_2 tensions (20%, 10% and 1%). Echinomycin, a functional blocker of HIF1α was applied to evaluate the HIF1α effects. In contrast to previous reports utilizing steroidogenic models, the cells were subjected to continuous hypoxia within a controlled hypoxia workstation, ensuring no oxygen bursts occurred during manipulation of cells. The stabilization of cells in hypoxia was initiated 24 hours before treatments. Various contrasts were set to assess the impact of cAMP, the influence of O2 on cAMP-mediated responses, and the effects of HIF1α. Differentially expressed proteins (DEPs) were used for downstream functional enrichment analysis ($P<0.05$, $FDR<0.01$). While, compared with their controls, cAMP at both 20% and 10% O_2 upregulated proteins involved in cholesterol metabolism, the cAMP treated cells at 1% O_2 downregulated proteins involved in mitochondrion organization, mitochondrial transport and NADH dehydrogenase complex assembly, biological processes essential for steroidogenesis. The contrast 10% vs 20% O_2 after cAMP treatment showed DEPs involved in cytokine secretion and transmembrane transporter activity, indicating highly responsive oxygen sensors. On the other hand, the contrast 1% vs 20% O_2 after cAMP treatment resulted in 166 DEPs, upregulating cytokine secretion-related factors, and downregulating, i.a., Hippo signaling-related factors and sialylation, as well as modulating activity of kinases. Interestingly, echinomycin decreased essential proteins involved in ribonucleoprotein complex biogenesis, indicating a possible role of HIF1α in global protein synthesis within steroidogenic cells. In a parallel study, the functional involvement of HIF1α in STAR-mediated steroidogenesis was confirmed by applying water-soluble (22R)-22-hydroxycholesterol to echinomycin-treated cells. This rescued progesterone production despite suppressed STAR expression. To optimize the verification of the newly discovered target molecules from our proteomics data, we designed a new 3D culture model for steroidogenic cells. The MA10 Leydig cells were plated in non-adherent 6 well plates for 48h, and resulting spheroids were assessed for viability, morphology and steroidogenic capacity. Histology revealed closely packed spheroids with high cell-density. The PCNA staining confirmed proliferation without signs of central necrosis, accompanied by low cleaved-caspase-3 expression, altogether indicating low apoptotic index. The spheroids responded to cAMP with increased STAR gene and protein expression and showed increased progesterone levels after treatment. In conclusion, our preliminary data offer new insights into hypoxia and HIF1α-dependent regulation of steroidogenesis, indicating functional targets of translational value. The

successful production of steroidogenic spheroids from the MA10 cell line could provide an alternative 3D *in-vitro* model for steroidogenic studies, one that might be more relevant for *in vivo* comparisons, and that will ultimately help broaden our understanding of the hypoxia-mediated effects in steroidogenic cells.

Supported by SNSF project nr. 310030_207895