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Hypoxia Inducible Factors and Associated MicroRNAs in Regulation of Steroidogenesis in the H295R Human Adrenocortical Carcinoma Cells

缺氧誘導因子及其相關的微小 RNA 在人類腎上腺皮質癌 H295R 細胞中對類固醇激素合成的調控

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ABSTRACT

The processes in living organisms that govern cellular adaptation and survival mechanisms to cope with hypoxic stress are complex and incompletely defined. Numerous in-vivo and in-vitro studies in mammals and non-mammalian vertebrates have shown that many steroidogenic activities and reproductive functions are impaired under chronic or acute hypoxic stress; however, the molecular basis for the reproduction impairments is still poorly known. The Hypoxia-Inducible Factors (HIFs) are a family of transcription factors that mediate many of the molecular responses to hypoxia, and over 100 genes controlling diverse cellular and physiologically processes are now known to be regulated by HIF proteins. Endogenous microRNA (miRNA) molecules (which are short non-coding RNAs with the ability to regulate gene expression post-transcriptionally) have been identified as essential mediators of numerous cellular processes, including responses to hypoxia. In particular, microRNA-210 (miR-210) is known to be specifically induced by HIF-1 during hypoxia. The cell-cycle regulator E2F3, the receptor tyrosine kinase ligand ephrin A3, and the DNA repair protein RAD52 are repressible gene targets of miR-210. However, the roles of the HIF family of proteins and related miRNAs in the regulation of steroidogenesis and reproductive functions have yet to delineated.

In this study, the hypothesis that HIFs and associated miRNAs are involved in regulating genes that control the steroidogenesis pathway in vertebrates was tested using the steroidogenic human H295R (adrenocortical carcinoma) cell line as a model. H295R cells express all of the key enzymes involved in steroidogenesis and have the ability to produce steroid hormones representative of the three distinct zones − zona glomerulosa, zona fasciculata and zona reticularis − in the adult adrenal cortex. Experiments were performed to determine the effects of hypoxia on the expression levels of: (1) HIF-1α, -2α and -3α by qRT-PCR and Western blot analyses; (2) nine

steroidogenic enzyme genes (HMGR, StAR, CYP11A1, 3β-HSD2, CYP17A1, CYP21A2, 17β-HSD1, 17β-HSD4 and CYP19A1) by qRT-PCR; (3) four transcription factor genes (SF-1, Dax-1, Nur-77 and Cited-2) that control steroidogenesis by q-RT-PCR; and (4) two sex hormones (testosterone and estradiol) by ELISA assays in H295R cells.

The effects of overexpression and knockdown of the human HIF-1α, HIF-2α and HIF-3α proteins (using the lentiviral expression system by Invitrogen) on steroidogenesis and hormone levels in H295R cells were also examined using the techniques described above. In addition, micro-RNA profiling experiments on normoxic, hypoxic, and HIFα-overexpressing and HIF-knockdown H295R cells were performed. Following extensive computational analyses, two miRNAs − miR-210 and miR-98 − were selected for further experiments. Overexpression and knockdown experiments of these two miRNAs were investigated in H295R cells, and their effects on the expression patterns of the nine steroidogenic genes and four regulatory factors (SF-1, Dax-1, Nur-77 and Cited-2); HIF-1α and HIF- 2α mRNAs; and testosterone and estrogen levels were determined.

Hypoxic H295R cells showed significant induction of the HIF-1α and HIF-2α proteins; but HIF-3α mRNA and protein were not detectable in H295R cells under normoxic and hypoxic conditions. Hypoxia differentially regulated expression of the nine steroidogenic enzyme genes, whereby downregulation of StAR, 17β-HSD1, 17β-HSD4 and CYP19 was observed. Expression of SF-1 and Dax-1 were reduced under hypoxia, while Nur-77 mRNA level was unaffected. Importantly, testosterone and estradiol levels were significantly reduced in hypoxic H295R cells.

CYP17A1 expression was downregulated while 17β-HSD1, CYP19A1, Dax-1 and Nur-77 were upregulated in HIF-1α knockdown cells, an observation opposite to that in hypoxic H295R cells, which strongly indicated that HIF-1α is likely

involved in the regulation of these genes. As compared to hypoxic cells, HIF-1α knockdown cells did not show a reduction in the two sex steroid hormones, which suggests the presence of another level of regulation. CYP21A2, CYP19A1 and Dax-1 genes were downregulated in the HIF-2α overexpressing cells (under normoxia) but upregulated in the HIF2-knockdown cells (under hypoxia), which suggested that these steroidogenic genes are likely regulated by HIF-2. 3β-HSD2 and CYP17A1 were also affected in the HIF-2α knockdown where the level of estradiol was decreased, while testosterone level was marginally increased. Overall, steroidogenic enzyme genes were observed to be differentially regulated by HIFs. Some steroidogenic enzyme genes such as CYP19A1 and 17β-HSD1 were found to be commonly affected by hypoxia and HIFs. Computational analysis of the 5′-flanking regions of these genes revealed several putative hypoxia responsive elements (HREs). This suggests that HIFs may be regulating these genes directly by binding to the HREs.

miRNA profiling studies showed that some 29% of the 379 human miRNAs that are expressed in normoxia showed altered expression under hypoxia. When hypoxically upregulated miRNAs were compared to those of HIF2α-overexpressing and HIF3α-overexpressing H295R cells, 44, 65 and 41 miRNAs, respectively, were found to be upregulated specifically in hypoxia, HIF-2α and HIF-3α-overexpressing cells. In contrast, 24, 23 and 10 miRNAs, respectively, were found to be co-upregulated in hypoxic and HIF3α-overexpressing cells, hypoxic and HIF2α-overexpressing cells, and HIF2α- and HIF3α-overexpressing cells. 16 miRNAs were co-upregulated in hypoxic, HIF1α-overexpressing, HIF2α-overexpressing and HIF3α-overexpressing cells. On the other hand, 21, 29 and 47 miRNAs, respectively, were found to be downregulated specifically under hypoxia, in HIF2α- and HIF3α-overexpressing H295R cells. In contrast, 5, 18, 11 miRNAs, respectively, were found to be co-downregulated in hypoxic and HIF2α-overexpressing cells, HIF2α- and

HIF3α-overexpressing cells, and hypoxic and HIF3α-overexpressing H295R cells. 16 miRNAs were downregulated in hypoxic, HIF1α-overexpressing, HIF2α-overexpressing and HIF3α-overexpressing cells. miRNA profiles in HIF-1α knockdown (under hypoxia) when compared to hypoxic H295R cells (to detect HIF-1α regulated miRNAs) showed that 26 miRNAs exhibited opposite expression patterns. Comparison of HIF2α-overexpressing and HIF2α-knockdown miRNA profiles showed 55 miRNAs are HIF-2α regulated.

Overexpression studies on miR-210 showed significant reduction in the mRNA levels of StAR and CYP17A1; while mRNA of StAR was only marginally upregulated and CYP17A1 remained unchanged in miR-210 knockdown cells. miR-210 overexpression significantly upregulated Nur-77 expression whereas Cited-2 was reduced. miR-210 overexpression increased estradiol and testosterone levels although the knockdown cells showed no change in these two hormones. Overexpression of miR-98 reduced the mRNA levels of StAR, CYP11A1, CYP17A1 and CYP19A1. miR-98 knockdown had no effect on StAR and CYP19A1 but expression of 3β-HSD2, CYP11A1, CYP17A1 and 17β-HSD1 were downregulated. Additionally, miR-98 overexpression reduced the expression of Dax-1, Cited-2 and SF-1. Expression of SF-1 and Dax-1 was also found reduced in miR-98 knockdown cells. In agreement with the reduced expression of certain steroidogenic enzyme genes, a significant reduction in estradiol production was observed miR-98-overexpressing H295R cells. Computer analysis showed that CYP19A1 is a likely gene target of miR-98. To verify whether the putative miR-98 binding site in the 3'-UTR of CYP19A is indeed functional, further investigations are needed.

Overall, this study describes the likely roles of HIFs in controlling steroidogenesis through the possible actions of specific miRNAs, and provides the basis for an alternative pathway through which steroidogenesis is modulated under

hypoxia. This study has provided some important insights into the relationships between hypoxia, HIFs, miRNAs and steroidogenesis.

TABLE OF CONTENTS