Hypoxia-inducible Factor-2α (HIF-2α) Is Involved in the Apoptotic Response to Hypoglycemia but Not to Hypoxia*

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Deprivation of oxygen (hypoxia) and/or glucose (hypoglycemia) represents a serious stress that affects cellular survival. The hypoxia-inducible transcription factor-1α (HIF-1α), which has been implicated in the cellular response to hypoxia (Semenza, G. L. (1999) Annu. Rev. Cell Dev. Biol. 15, 551–578), mediates apoptosis during hypoxia (Halterman, M. W., Miller, C. C., and Federoff, H. J. (1999) J. Neurosci. 19, 6818–6824 and Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P., Moons, L., Jain, R. K., Collen, D., and Keshet, E. (1998) Nature 394, 495–499), but the function of its homologue HIF-2α remains unknown. Therefore, the role of HIF-2α in cellular survival was studied by targeted inactivation of the HIF-2α gene (HIF-2α−/−) in murine embryonic stem (ES) cells. In contrast to HIF-1α deficiency, loss of HIF-2α did not protect ES cells against apoptosis during hypoxia. Both HIF-1α−/− and HIF-2α−/− ES cells were, however, resistant to apoptosis in response to hypoglycemia. When co-cultured with wild type ES cells, HIF-2α−/− ES cells became rapidly and progressively enriched in hypoglycemia but not in hypoxia. Thus, HIF-1α and HIF-2α may have distinct roles in responses to environmental stress, and despite its name, HIF-2α may be more important in the survival response to environmental variables other than the level of oxygen.

HIF-1α is a basic helix-loop-helix transcription factor, which mediates the cellular adaptation to hypoxia (1, 3). During hypoxia, HIF-1α up-regulates the expression of a number of genes involved in erythropoiesis, glycolysis, and angiogenesis by formation of a heterodimer with HIF-1β, which binds to a hypoxia-response element in the promoter of these target genes (1, 3, 5–7). Several studies have shown that loss of HIF-1α or HIF-1β impaired gene expression in response to hypoxia and/or hypoglycemia (5–8), even though the precise molecular mechanisms for the latter condition remain largely undetermined. HIF-1α has also been implicated in the induction of apoptosis during stressful conditions of hypoxia and hypoglycemia (5).

Recently, a novel hypoxia-inducible factor, HIF-2α (also known as EPAS1* (9), HLF (10), HRF (11), or MOP2 (12)), was identified, which also binds as a heterodimer with HIF-1β to the hypoxia-response element. Like HIF-1α, HIF-2α is subject to oxygen-dependent proteosomal destruction, mediated by the von Hippel-Lindau tumor suppressor protein (13), and the protein levels of HIF-2α are increased under hypoxic conditions (14). Gene inactivation studies revealed that HIF-2α is essential for cardiovascular development and angiogenesis (15, 16), but it remains unknown whether these embryonic defects resulted from insufficient hypoxic up-regulation of target genes. In fact, expression of vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR-2/Flk-1), and the endothelial receptor Tie-2 was comparable in HIF-2α−/− and wild type embryos (16). Although ectopic overexpression of HIF-2α in vitro stimulates reporter gene expression in hypoxic conditions, the induction is variable and lower than that by HIF-1α, and surprisingly, HIF-2α already stimulates reporter gene expression in normoxia (9, 12, 14, 17, 18). Thus, the precise role of endogenous HIF-2α in mediating the cellular responses to hypoxia remains uncertain. In addition, the role of HIF-2α as compared with HIF-1α in apoptotic processes remains to be elucidated. Because HIF-dependent gene regulation is emerging as a target for anti- or proangiogenic treatments (19, 20), it is important to define the role of HIF-2α. In the present study, the endogenous role of HIF-2α in cellular survival was examined by targeted gene inactivation in murine embryonic stem (ES) cells.

EXPERIMENTAL PROCEDURES

Generation of Targeted ES Cell Clones—ES cells deficient in HIF-1α (HIF-1α−/−) were generated previously (5). The HIF-2α targeting vector pNPT.HIF-2α (Fig. 1A) contained a 5′-flanking 3.5-kb HindIII/StuI fragment upstream of exon 2 and a 3′-flanking 7.0-kb fragment (a 2- kb Nhel/BomHI fragment and an immediately downstream 5-kb BomHI/ EcoRI fragment) downstream of exon 2. Culture and targeting of undifferentiated ES cells, including selection in high G418 and Southern blot analysis, were done as described (5). Inactivation of the HIF-2α gene was confirmed by RT-PCR and immunoblot analysis. Forward
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primer 5'-ACGGAAGCTCCAGGTCCCTGTGCTGCTG3' (exon 1) and reverse primer 5'-ACAGAGGAGCTTTATGTGCTCGAAGGGAGG3' (exon 2) were used for RT-PCR to amplify a 531-bp HIF-2α-specific fragment. A 374-bp HPRT-specific fragment (internal control) was amplified by RT-PCR using forward primer 5'-GTCGCCCTCTGTGCTGCTAAGAGGGG3' and reverse primer 5'-AAACTTGTCTGGAATTTCAAATCC3'. Protein extraction and immunoblot analysis were performed as described previously (14). Monospecific polyclonal antibodies (PM 8) against murine HIF-2α were generated and purified using affinity columns (21).

Hypoxic and Hypoglycemic Treatment of ES Cells and Apoptosis Measurements—ES cells were seeded at low cell density (10,000 cells/35-mm culture dish) and cultured in medium supplemented with 20% fetal bovine serum and 5% fetal calf serum (FCS) for 24 h. Afterward, ES cells were stressed in the same medium without leukemia inhibitory factor and 5% fetal calf serum (FCS) for 24 h. Subsequently, stressed ES cells were allowed to recover for 4 h in normoxia/normoglycemia (medium containing 15% FCS). Thereafter, ES cells were split, cultured until they reached 30% confluency, and challenged with another cycle of 20 h of stress. At every split, 300,000 ES cells were replated in a 24-well plate, subcultured for 24 h, and subsequently fixed with 2% formaldehyde, 0.5% glutaraldehyde. After staining for β-galactosidase as described (15), the percentage of HIF-2α-positive cells in the mixed populations was quantified using the Scion Image 1.60c system (Meyer Instruments, Inc., Houston, TX).

Quantitative Real Time RT-PCR Analysis—Gene expression of Glut-1, PK-1, VEGF, Flk-1, Flt-4, and Tie-2 was quantified by Real Time RT-PCR, relative to the expression level of HPRT, using the following forward (F) and reverse primers (R) and probes (P), labeled with fluorescent dye (FAM or JOE) and quencher (TAMRA) as previously described (23). For Glut-1: 5'-GGGGCGTGTTCCCTCAGTATGTG3' (exon 1) and 5'-GAGGAGGACCCCATCGAAGAT3' (exon 2); for PK-1: 5'-GGGGCGTGTTCCCTCAGTATGTG3' and 5'-GAGGAGGACCCCATCGAAGAT3'; for VEGF: 5'-AGTCCGCAATGATGAAATCG3' and 5'-ACGAGGACCCCATCGAAGAT3'; for Flk-1: 5'-GGGGCGTGTTCCCTCAGTATGTG3' and 5'-GAGGAGGACCCCATCGAAGAT3'; for Flt-4: 5'-GGGGCGTGTTCCCTCAGTATGTG3' and 5'-GAGGAGGACCCCATCGAAGAT3'; for Tie-2: 5'-GGGGCGTGTTCCCTCAGTATGTG3' and 5'-GAGGAGGACCCCATCGAAGAT3'; for HPRT: 5'-GGGGCGTGTTCCCTCAGTATGTG3' and 5'-GAGGAGGACCCCATCGAAGAT3'.

RESULTS AND DISCUSSION
Targeted inactivation of a single HIF-2α allele (HIF-2α+/−) in ES cells was accomplished by homologous deletion of the second exon, encoding the basic helix-loop-helix domain, essen-

- **Fig. 1. Targeting of the HIF-2α gene.** A, top, targeting vector pPNT.HIF-2α; middle, map of the WT gene; bottom, homologously recombined HIF-2α allele (HIF-2α+/−). Restriction digests and hybridization probes A (0.6 kb NcoI-EcoRV fragment) and B (2.3 kb XhoI-EcoRI fragment) are indicated. B, Southern blot analysis (probe A) of StuI-digested genomic DNA from WT (+/+), heterozygous-deficient HIF-2α+/− and homozygous-deficient HIF-2α−/− ES cells generating a 7-kb WT and 7.5-kb homologously recombined HIF-2α allele. C, RT-PCR analysis of total RNA of WT (+/+) and HIF-2α−/− ES cells for HIF-2α gene expression. HPRT gene expression was used as an internal control. D, immunoblot analysis on total cell extract from WT (+/+) and HIF-2α−/− ES cells for HIF-2α and HIF-1α gene expression during normoxia (N) and hypoxia (H). HIF-2α protein was undetectable under normoxia. Only WT ES cells cultured in hypoxia show the presence of HIF-2α protein. The faint residual bands represent background that cross-reacted in a nonspecific way.
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Table I

| Apoptosis in WT, HIF-1α−/−, and HIF-2α−/− ES cells |
|-----------------------------------------------|
| Data represent means ± S.D. (n = 9–12) of the number of oligonucleosomes, the picogram amounts of p53 antigen, or the units of Bcl-2, all per 10⁶ cells. |

| Oligonucleosomes          | Normoxia/normoglycemia | Hypoxia                  | Hypoglycemia         | Interleukin-1β/IFN-γ/TNF-α |
|---------------------------|-------------------------|--------------------------|----------------------|---------------------------|
| WT                        | 45 ± 4                  | 320 ± 26*                | 350 ± 32*            | 370 ± 32*                 |
| HIF-1α−/−                 | 37 ± 3                  | 39 ± 5                   | 41 ± 2               | 370 ± 22*                 |
| HIF-2α−/−                 | 44 ± 4                  | 331 ± 32*                | 51 ± 8               | 400 ± 51*                 |
| p53                       | WT                      | 0.14 ± 0.05              | 3.3 ± 0.1*           | 2.9 ± 0.3*                |
| HIF-1α−/−                 | 0.12 ± 0.07             | 0.13 ± 0.07              | 0.16 ± 0.03          | 3.6 ± 0.7*                |
| HIF-2α−/−                 | 0.18 ± 0.06             | 3.1 ± 0.3*               | 0.18 ± 0.07          | 3.9 ± 0.9*                |
| Bcl-2                     | WT                      | 120 ± 15                 | 21 ± 4*              | 17 ± 1*                   |
| HIF-1α−/−                 | 120 ± 5                 | 120 ± 4                  | 130 ± 13             | 31 ± 5*                   |
| HIF-2α−/−                 | 130 ± 27                | 38 ± 11*                 | 140 ± 16             | 39 ± 5*                   |

*Statistically significant (p < 0.05) versus control (WT/normoxia).

Fig. 2. Enrichment of HIF-2α−/− ES cells by hypoglycemia. A, LacZ-staining was performed on the starting culture containing 5% HIF-2α−/− and 95% WT ES cells. B, after 13 cycles of hypoglycemic stress the LacZ-positive HIF-2α−/− ES cells have outnumbered the WT ES cells completely. C and D, after 13 passages in normoxia (C) or 13 cycles of hypoxic stress (D) the enrichment of HIF-2α−/− ES cells was much smaller. E, control LacZ stainings on pure HIF-2α−/− ES cells (at start). F, pure WT ES cells (after 13 cycles of hypoglycemic stress) did not stain for LacZ, excluding that LacZ staining in the mixed cultures was due to endogenous expression of β-galactosidase of WT ES cells. G, graphic representation of the enrichment of HIF-2α−/− ES cells in mixed cell populations under normoxia, hypoxia, and hypoglycemia shows that HIF-2α−/− ES cells have a survival advantage when compared with WT ES cells specifically in hypoglycemia but not in hypoxia. H, I, and J, bar graphics, presenting the percentage of WT (+/+), and LacZ-positive HIF-2α−/− ES cells (−/−) in mixed cell populations, indicate the fast and progressive enrichment of HIF-2α−/− ES cells under hypoglycemia (J) but not under normoxia (H) or hypoxia (I).

tial for DNA binding and dimerization of HIF-2α with HIF-1β to form a functional HIF2 complex (1, 3, 9) (Fig. 1, A and B). HIF-2α−/− ES cells were obtained by selection of HIF-2α−/− ES cells in high G418. Functional inactivation of the HIF-2α gene was confirmed by RT-PCR and immunoblot analysis, using monospecific antibodies against murine HIF-2α (Fig. 1, C and D). Control immunoblots using antibodies against HIF-1α protein confirmed the residual expression of HIF-1α in HIF-2α−/− ES cells (data not shown). ES cell clones with a randomly integrated HIF-2α gene targeting vector and with two functional HIF-2α alleles (HIF-2α+/−) were used as controls (further referred to as WT).

We have previously demonstrated that apoptosis of ES cells in response to hypoxia and hypoglycemia is mediated by HIF-1α via a mechanism involving up-regulation of p53 and down-regulation of Bcl-2 (5). The role of HIF-2α in cellular apoptosis during hypoxia was compared with that of HIF-1α by measuring the number of oligonucleosomes (or TUNEL-positive cells; similar results; not shown), which are liberated during apoptosis. WT, HIF-1α−/−, and HIF-2α−/− ES cells did not undergo apoptosis under normal conditions (25 mM glucose and 20% oxygen; Table I). During hypoxia (0.5% O₂) or hypoglycemia (0 mM glucose), apoptosis significantly increased in WT ES cells but, as previously reported (5), not in HIF-1α−/− ES cells (Table I). In contrast, HIF-2α−/− ES cells were refractory to hypoglycemia but, surprisingly, not to hypoxic stress (Table I). The role of HIF-1α and HIF-2α in hypoxia and hypoglycemia was specific, because serum deprivation (not shown) and stimulation with Fas ligand (not shown) or a cytokine-mixture (IL-1β/IFN-γ/TNF-α) comparably stimulated apoptosis in WT, HIF-1α−/−, and HIF-2α−/− ES cells (Table I). When apoptosis occurred, protein levels of p53 (a mediator of genotoxic apoptosis, up-regulated during hypoxia) were up-regulated, whereas those of Bcl-2 (an apoptosis inhibitor) were reduced (Table I). These data indicate that, in contrast with HIF-1α, HIF-2α specifically induces up-regulation of p53 and down-regulation of Bcl-2 in ES cells under hypoglycemia but not under hypoxia. To confirm the role of HIF-2α in the control of ES cell survival during hypoglycemia, we studied whether HIF-2α−/− ES cells would exhibit a survival advantage and become enriched over WT ES cells when both cell types were co-cultured in hypoglycemia. Therefore, HIF-2α−/− and WT ES cells were
mixed at a ratio of 1:20 (5% HIF-2α−/− and 95% WT ES cells) (Fig. 2A) and cultured for repetitive cycles of 20-h hypoglycemia or hypoxia alternating with 28-h normoxia/normoglycemia. To distinguish HIF-2α−/− from WT ES cells, the former were stably transfected with a β-galactosidase gene (allowing easy detection after LacZ staining), whereas the latter were mock-transfected. Pools of HIF-2α−/− and WT ES cells were used to avoid clonal selection. When each of these cell types was grown separately in normoxia/normoglycemia, HIF-2α−/− ES cells grew slightly faster than WT cells (doubling time: ~15 h for HIF-2α−/− cells versus 16 h for WT cells). However, when the mixture of both cell types was intermittently and repetitively challenged by hypoglycemia (20-h hypoglycemia followed by 28-h normoxia/normoglycemia), HIF-2α−/− ES cells became progressively enriched over WT cells, and after 13 cycles of hypoglycemic stress, WT cells were overgrown and absent from the culture dish as revealed by LacZ staining (Fig. 2, B, G, and J). In contrast, intermittent hypoxia or normoxia failed to enrich LacZ-positive HIF-2α−/− ES cells within 13 cycles (Fig. 2, C, D, G, H, and J). Because of their slightly faster growth potential (see doubling times above), HIF-2α−/− ES cells ultimately also became enriched in hypoxia or normoxia/normoglycemia but only after many more cycles of hypoxia (n = 33) than of hypoglycemia (n = 13) (Fig. 2, G–J). This is not surprising considering the slightly faster intrinsic growth rate of HIF-2α−/− cells in normoxia/normoglycemia, e.g. any cell type with a slightly shorter doubling time will overgrow another more slowly growing cell population, even if the difference in doubling time is minimal. Importantly, however, enrichment of HIF-2α−/− cells required a comparable number of passages in normoxia and hypoxia, indicating that these cells had no survival advantage over WT cells under hypoxia. In contrast, under hypoglycemia, the enrichment of HIF-2α−/− cells occurred much faster than in normoxic or hypoxic conditions (Fig. 2G), indicating that these cells indeed had a survival advantage over WT cells in hypoglycemic conditions. Similar results were obtained when different ratios of HIF-2α−/− and WT ES cells were mixed and challenged (not shown). Thus, the fast and progressive enrichment of HIF-2α−/− ES cells in hypoglycemia but not in hypoxia is consistent with their refractoriness to hypoglycemia-induced apoptosis and with the minimal involvement of HIF-2α in hypoxia-induced apoptosis.

To examine whether HIF-1α and HIF-2α always had a distinct role in mediating the response to hypoxia and hypoglycemia, we determined by quantitative Real Time RT-PCR the expression levels of known HIF-1α target genes in ES cells under normoxia, hypoxia, and hypoglycemia. The role of HIF-1α and HIF-2α in controlling gene expression was found to be dependent on the specific target gene (Fig. 3). Similar to its role in regulating cell survival, HIF-2α controlled expression of the glucose transporter-1 (Glut-1) in hypoglycemia but not in hypoxia, whereas HIF-1α up-regulated its expression in both conditions, consistent with previous findings (1, 2, 5–7). In contrast, expression of phosphoglycerate kinase-1 (PGK-1) in hypoxia and hypoglycemia was only dependent on HIF-1α but not on HIF-2α, whereas induction of VEGF and Flk-1 transcripts was dependent on both HIF-1α and HIF-2α (Fig. 3, A–D). The role of HIF-2α in hypoxic gene expression of VEGF (10, 14, 24) and Flk-1 (17) confirms previous findings. HIF-2α induced expression of Fli-4/VEGFR-3 in hypoglycemia and hypoxia, whereas HIF-1α only up-regulated its expression in hypoglycemia. Expression of the endothelial receptor Tie-2 was not induced by hypoxia, but its up-regulation under hypoglycemia was more dependent on HIF-2α than HIF-1α (Fig. 3, E–F). Previous studies also observed that reporter gene expression under control of the Tie-2 promoter was only induced by HIF-2α but not by HIF-1α (9). Taken together, these findings indicate that HIF-1α and HIF-2α have differential and distinct roles in biological processes such as apoptosis, angiogenesis, and glycolysis dependent on the particular target gene and stress condition.

In conclusion, these genetic data indicate that HIF-1α and HIF-2α have distinct roles in the adaptation of the cellular response to deprivation of oxygen or nutrients. Whereas HIF-1α is involved in regulating the expression of both angiogenic factors (VEGF, Flk-1) and glycolytic enzymes (Glut-1, PGK-1), HIF-2α is more restricted to the regulation of angiogenic processes but is only minimally involved in glycolysis. In addition, this study provides evidence that HIF-2α may act more as a hypoglycemia than a hypoxia response factor in apoptotic processes. How HIF-2α is involved in the cellular response to hypoglycemia remains to be unraveled because HIF-2α protein does not accumulate during hypoglycemia, suggesting that the latter condition activates HIF-2α via mechanisms distinct from those whereby hypoxia activates HIF-2α and HIF-1α (1, 3, 13, 14). Our data indicate that gene induction by either HIF-1α and/or HIF-2α may not only depend on the
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particular target gene but also on the type of stress. Because the expression pattern of HIF-1α and HIF-2α in vivo is distinct (9–11, 25), gene induction by a particular HIF is likely to depend on the cell type.

The role of HIF-2α in the cellular response to hypoglycemia might be significant for a number of biological processes and pathological disorders. For instance, hypoglycemia may cause congenital cardiac (26) and neural malformations (27). In preterm or small for gestational age infants, hypoglycemia causes cerebral damage and edema (28) and impairs psychomotor development (29), in particular in children of diabetic mothers (30). Glucose levels are often undetectable in tumors (31), whereas neurons in Alzheimer’s disease have impaired glucose uptake, causing intracellular glucose deprivation (32). Non-coma hypoglycemia or stroke may also induce neuronal apoptosis (33). Because all these processes are characterized by glucose deprivation in tissues, known to express neuronal apoptosis (33), gene induction by a particular HIF is likely to remain whether HIF-2α is involved in the pathogenesis of these disorders, and if so, whether modulation of its activity might bear any therapeutic potential.

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