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Non-hypoxic induction of HIF-3α by 2-deoxy-D-glucose and insulin

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Abstract

Hypoxia-inducible factors (HIFs) are key mediators of cellular adaptation to hypoxia, but also respond to non-hypoxic stimuli. To clarify involvement in metabolic disturbances, HIFs were characterised in rats subjected to insulin-induced hypoglycaemia or cellular glucoprivation provoked by 2-deoxy-D-glucose (2-DG). Using real-time qPCR, organ-specific expression of HIF-1α, -2α, -3α, -1β, and of the target gene GLUT-1 was determined. Distribution of HIF-3α proteins was examined by immunohistochemistry. Both, insulin and 2-DG resulted in a widespread increase in HIF-3α mRNA. HIF-2α mRNA increased in lung and heart after 2-DG only, whereas other HIFs remained unaffected. A pronounced increase of protein levels in cerebral cortex was observed for HIF-3α. Functional significance of HIF induction was reflected in enhancement of GLUT-1 mRNA. Transcriptional up-regulation of HIF-3α represents a typical response to *in vivo* hypoglycaemia and glucoprivation. These data suggest an involvement of the HIF system in metabolic derangements as for instance caused by diabetes.

Keywords: 2-Deoxy-D-glucose; Gene expression; Glucoprivation; Hypoglycaemia; Hypoxia-inducible factors; Rats

Metabolic diseases are frequently associated with derangements of glucose homeostasis which ultimately could result in depletion of intracellular energy supplies. This loss activates mechanisms that adjust glucose availability, e.g., by induction of glycolysis. Little is known regarding a potential cross-talk between blood glucose control and hypoxia-inducible factors (HIFs) during adaptation to imbalanced metabolic conditions. Very recent data indicates that not only insulin [1] but also hyperglycaemia has potential to regulate a major component of this system, HIF-1α [2]. However, the *in vivo* influence of metabolic derangements, in particular hypoglycaemia and cellular glucose depletion, on HIFs is unclear.

HIF-1α belongs to a system consisting of basic-helix–loop–helix proteins (bHLH) Per-ARNT-Sim (PAS) domain master transcriptional regulators that are particularly involved in maintaining oxygen homeostasis at the cellular level and therefore improve cellular and systemic survival (for review see [3]). To this day, the existence of four HIF subunits has been confirmed, three representing α-class subtypes, HIF-1α, -2α, and -3α, and one representing the β-class subunit, HIF-1β [4,5]. Vital processes such as energy metabolism, angiogenesis, or erythropoiesis are driven by HIF-dependent target genes [3]. Many of these target genes are involved in the regulation of glucose metabolism, and especially glycolysis. Specific examples include the genes that encode enolase 1, lactate dehydrogenase A [6], and hexokinase II [7]. In addition, HIF target genes induce mechanisms that increase cellular glucose uptake, i.e., the glucose transporters GLUT-1 and GLUT-3 [8].

In contrast to the extensively studied HIF-1α, the knowledge about function and regulation of HIF-3α is fragmentary, but this subtype does appear to be adorned with strikingly different features. In contrast to the other HIF-α subunits, short-term hypoxia *in vivo* rapidly

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increased levels of HIF-3α mRNA, indicating a regulation of this subunit at the level of mRNA expression [9]. Regarding the physiological function, there are hints that HIF-3α might suppress HIF-1α/-2α induced gene expression [10]. The relevance of these multiple variants still needs to be determined *in vivo*. Recently, a protein with structural similarity to HIF-α subunits was discovered in mice that was capable of dimerising HIF-1α. The resulting complex did not bind to the HRE, and therefore inhibited HIF driven target gene expression. This factor was later termed inhibitory PAS protein (IPAS) [11]. In subsequent studies, IPAS was identified as an alternatively spliced variant of the murine HIF-3α locus [12]. This antagonist of HIF-1α transcriptional activity probably promotes suppression of neovascularisation in tissues with physiologically low oxygen tensions such as the corneal epithelium [11].

In view of the significance of the HIF system in glucose homeostasis, we studied the glucose-dependency of HIF expression patterns and changes in transcriptional regulation of HIF target genes in organs by simulating cellular energy depletion. This was induced by either hypoglycaemia or intracellular glucoprivation *in vivo* with respect to the fact that the latter condition is thought to be a promising tool in fighting various kinds of cancer [13].

Materials and methods

HT-22 cell culture. HT-22 mouse hippocampal cells (generously provided by Drs. C. Behl, Mainz, Germany and D. Schubert, La Jolla, USA) were cultured (37 °C, 5% CO_2) in Dulbecco's modified Eagle's medium containing 10% FCS (Biochrom), 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories).

Animals. Animal studies were approved by the 'Ministerium für Natur und Umwelt des Landes Schleswig-Holstein', Germany. Male Wistar rats (280–300 g; Charles-River) were housed in plastic cages under a 12 h light-dark cycle and had free access to standard chow and water. Animals were anaesthetised (pentobarbital 50 mg/kg i.p.) and a jugular vein indwelling catheter (Smiths Medical) was implanted.

Induction of hypoglycaemia and glucoprivation. Four days after surgery, four groups of animals received either insulin (1.5 U/kg; NovoNordisk), 2-deoxy-D-glucose (2-DG, 150 mg/kg; Sigma), or corresponding control treatments ($n = 5$–7). Substances were applied three times i.v., at intervals of 2 h as boli (Fig. 1). Rats were killed ten minutes after the last infusion to maintain the actual expression status of the parameters of interest. HT-22 cells were treated with 2-DG (20 mmol/l) in the presence of high (4.5 mg/ml) or low (1 mg/ml) glucose levels in the culture medium ($n = 15$).

Tissue preparation. Cerebral cortex, hypothalamic tissue, lung, heart, liver, and kidney were dissected, snap frozen in liquid nitrogen and stored at −80 °C. Since HIF proteins are known to be degraded within minutes, organs for immunohistochemical staining were perfused *in situ* with buffered formalin (4% pH 7.4, Sigma) to assure rapid fixation followed by a standard routine processing and embedding of the samples in paraffin using an automatic tissue processor (Leica TP 1020). Embedded tissues were sectioned into 2 μm slices using a microtome (HM440E, Micron).

Plasma parameters. Blood samples were centrifuged and plasma was kept at −20 °C. Plasma samples were assayed for glucose by the glucose oxidase method (Ascensia Elite® XL, Bayer Vital), for lactate enzymatically by the PAP method (AEROSET™ Analyzer, Abbott) and for insulin levels by a rat insulin-specific RIA (Linco).

RNA isolation and cDNA synthesis. Total RNA was isolated from tissue homogenates and cell lysates by the guanidinium isothiocyanate method, treated with DNase I, and purified using silica-gel-based spin columns (RNeasy Kit, Qiagen). First strand cDNA was synthesised from 1 μg of total RNA in the presence of 5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTPs, 1 U/μl RNasin, 0.5 μg oligo-$(dT)_{15}$ primer, and 15 U AMV reverse transcriptase (Promega).

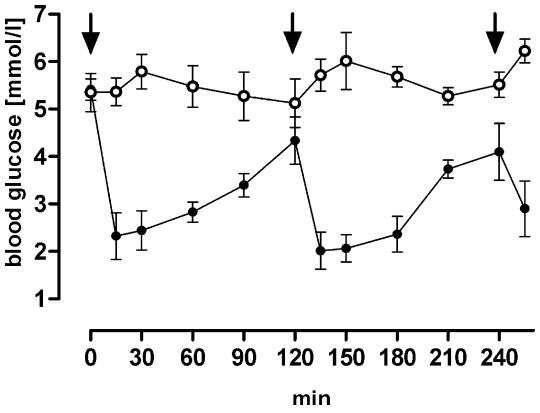

Fig. 1. Plasma glucose levels in Wistar rats during treatment with saline (○) or insulin (●). Arrows indicate dates of infusions. Data presented as means ± SEM.

Quantitative real-time PCR. Oligonucleotide sense and antisense primers specific for HIF-1α, -1β, -2α, -3α, GLUT-1, and EPO were synthesised as published previously [9]. As described elsewhere [14], real-time PCR was performed in triplicate using SYBR green I (Eurogentec) on a GeneAmp 7000 sequence detection system (Applied Biosystems) and quantified using the cycle threshold (C_T) method. Product purity was confirmed by dissociation curve analysis and agarose gel electrophoresis.

Protein analysis. Immunohistochemical analysis was performed as published previously [15]. Sections were incubated either with anti-HIF-1α, anti-HIF-2α (Novus Biologicals), or anti-HIF-3α antiserum (Santa Cruz Biotechnology) for 15 min at room temperature. A corresponding blocking peptide (Santa Cruz Biotechnology) was used for competition studies and quality control. Primary antibodies were linked (anti-mouse, anti-rabbit, or anti-goat antiserum) to a catalysed signal amplification system (CSA-System, Dakocytomation). Development was carried out using 3-amino-9-ethylcarbazole (AEC plus, Dakocytomation) followed by counterstaining with haematoxylin. Photographs were digitally recorded (Axioplan 2 software, Carl Zeiss Vision). To ensure specificity of the procedure, controls included staining of tissues from untreated animals, omission of primary antibodies, and incubation of samples with isotype antibodies.

Immunoblotting of HIF-3α proteins were carried out as described previously [9] and performed three times as independent experiments using a high-resolution CCD camera (ChemiDoc system and Quantity One software, Bio-Rad). The specificity of the antibodies was confirmed in a coexisting study applying HIF-3α-specific silencing RNA that suppressed the specific band in the immunoblot (unpublished data). Equal loading was verified by incubation of the membranes with antibodies against actin (Santa Cruz Biotechnology).

Statistical analysis. Data are presented as means ± SEM. Differences among the experimental groups were evaluated using the unpaired Student's t test. $P < 0.05$ was considered statistically significant.

Results

Plasma data

Bolus infusions of insulin led to significant drops in plasma glucose levels toward a range of 2–2.5 mmol/l with slow recovery over the following 120 min to lower levels of plasma glucose of ~4 mmol/l compared to initial values

(Fig. 1). Venous levels of insulin in control subjects as assayed by RIA ranged within physiological levels (2.62 ± 0.4 ng/ml) and were not affected significantly by 2-DG (1.82 ± 0.1 ng/ml). Plasma lactate levels did not differ amongst the treatment groups and ranged within physiological norms (control 1.30 ± 0.1 mmol/l, insulin 1.69 ± 0.2 mmol/l, and 2-DG 1.42 ± 0.2 mmol/l) indicating that the intensity of the induced energy depletion was moderate.

HIF-1α, HIF-2α, HIF-3α, and HIF-1β mRNA levels during treatment with insulin or 2-DG

Basal expression of mRNAs encoding for HIF-α class proteins 1, 2, and 3 and the HIF-1β subunit, as well as the expression of the examined target genes EPO and GLUT-1 obtained from the actual experiments ranged within numbers that were observed and described in detail elsewhere [9]. HIF-1α and HIF-1β mRNA levels were not affected by either hypoglycaemia or cellular glucoprivation in all organs that were investigated (Fig. 2A). HIF-2α mRNA increased 2-fold in the lung and heart after application of 2-DG but was unaffected in other organs or after insulin treatment. In contrast, mRNA levels of HIF-3α were markedly influenced by treatment with both insulin and 2-DG. Rats receiving repetitive insulin infusions displayed a pronounced up-regulation of HIF-3α mRNA in the hypothalamus, lung, and myocardium. Treatment with 2-DG induced a marked increase in HIF-3α mRNA levels in all organs analysed. In detail, HIF-3α mRNA amounts rose about 320% in cortex, 330% in hypothalamus, 960% in lung, 900% in the heart, 410% in the kidney, and comparatively low but still significantly, 190% in the liver. Taken together, both applied non-hypoxic stimuli were effective in activating HIF-3α mRNA expression with 2-DG appearing to be the more potent trigger than insulin. This induction was also reproducible in HT-22 cells that were used to rule out indirect effects of 2-DG which may have occurred *in vivo* (Fig. 2B). Hence, in the presence of 1 mg/ml ('low') glucose concentrations, 2-DG approximately doubled HIF-3α mRNA expression. This effect was diminished when cells were maintained in medium with 4.5 mg/ml ('high') glucose concentrations indicating a competitive interaction between 2-DG and glucose. Glucose levels alone had no influence on HIF-3α mRNA levels.

Changes in mRNA levels of the HIF-dependent target genes GLUT-1 and EPO

To determine functional consequences of activation of the HIF system by hypoglycaemia and glucoprivation, GLUT-1 and EPO were chosen as representative HIF target genes. A moderate but significant increase in GLUT-1 mRNA levels during treatment with insulin only occurred in the lung (180%). Interestingly, GLUT-1 mRNA levels in the liver were slightly diminished (70%). Unlike insulin, application of 2-DG produced a significant rise of GLUT-1 mRNA amounts in samples of hypothalamic tissue (160%), lung (190%), heart (230%), and the liver (150%). Levels of EPO mRNA ranged constantly below detection limit. In summary, both treatment regimes induced transcription of the HIF target gene GLUT-1 with a distinct organ distribution. Again, induction of GLUT-1 was more pronounced under conditions of glucoprivation.

Analysis of HIF-3α protein expression

To evaluate the distribution of HIF-α proteins and consecutive changes during glucoprivation or hypoglycaemia, immunohistochemistry was performed in sections of cerebral cortex and kidney representatively. Staining with specific antibodies was performed in three independent experiments. Application of the corresponding control peptide completely abolished the signal representing HIF-3α (not shown). 2-DG as well as insulin led to an accumulation of HIF-1α and HIF-2α proteins in the kidney and the cerebral cortex (data not shown), thereby confirming corresponding observations with insulin in previous studies [1,16]. In animals receiving saline, HIF-3α was not detected in samples of kidneys whereas a weak basal expression of HIF-3α proteins was noted in the cerebral cortex, localised mainly in the cytoplasmic compartment of neuronal cells (Fig. 3A). In all samples examined, application of either 2-DG or insulin led to dense signals representing HIF-3α. In cerebral cortex, a pronounced staining was observed particularly in neuronal cells. Interestingly, hypoglycaemia and glucoprivation induced a preferentially perinuclear expression of HIF-3α proteins. In renal tissue, insulin treatment produced an intense accumulation of HIF-3α proteins with highest enhancement occurring in renal tubules and less in the glomeruli. Similar to the cerebral cortex, HIF-3α proteins were localised mainly around the cell nuclei.

Immunoblotting of HIF-3α protein obtained from 2-DG treated HT-22 cells that were cultured in the presence of low glucose levels revealed a significant increase of protein amounts indicating the activation of this HIF subunit by cellular depletion of glucose (Fig. 3B).

Discussion

As its main finding, this study demonstrated that the HIF system is inducible by non-hypoxic stimuli such as insulin-dependent hypoglycaemia and insulin-independent cytoglycopaenia. Moreover, it was demonstrated that these non-hypoxic triggers were fully effective to cause a rapid increase of HIF-3α mRNA levels, a phenomenon that appears to be a feature unique to this HIF subunit.

As noted above, 2-DG as an energy restriction mimetic agent has attracted increasing interest due to its potential usage as an antineoplastic agent with the ulterior motive that tumour cells are highly dependent on glycolysis to serve their metabolic requirements [13,17]. 2-DG, a structural analogue of glucose that differs at the second carbon

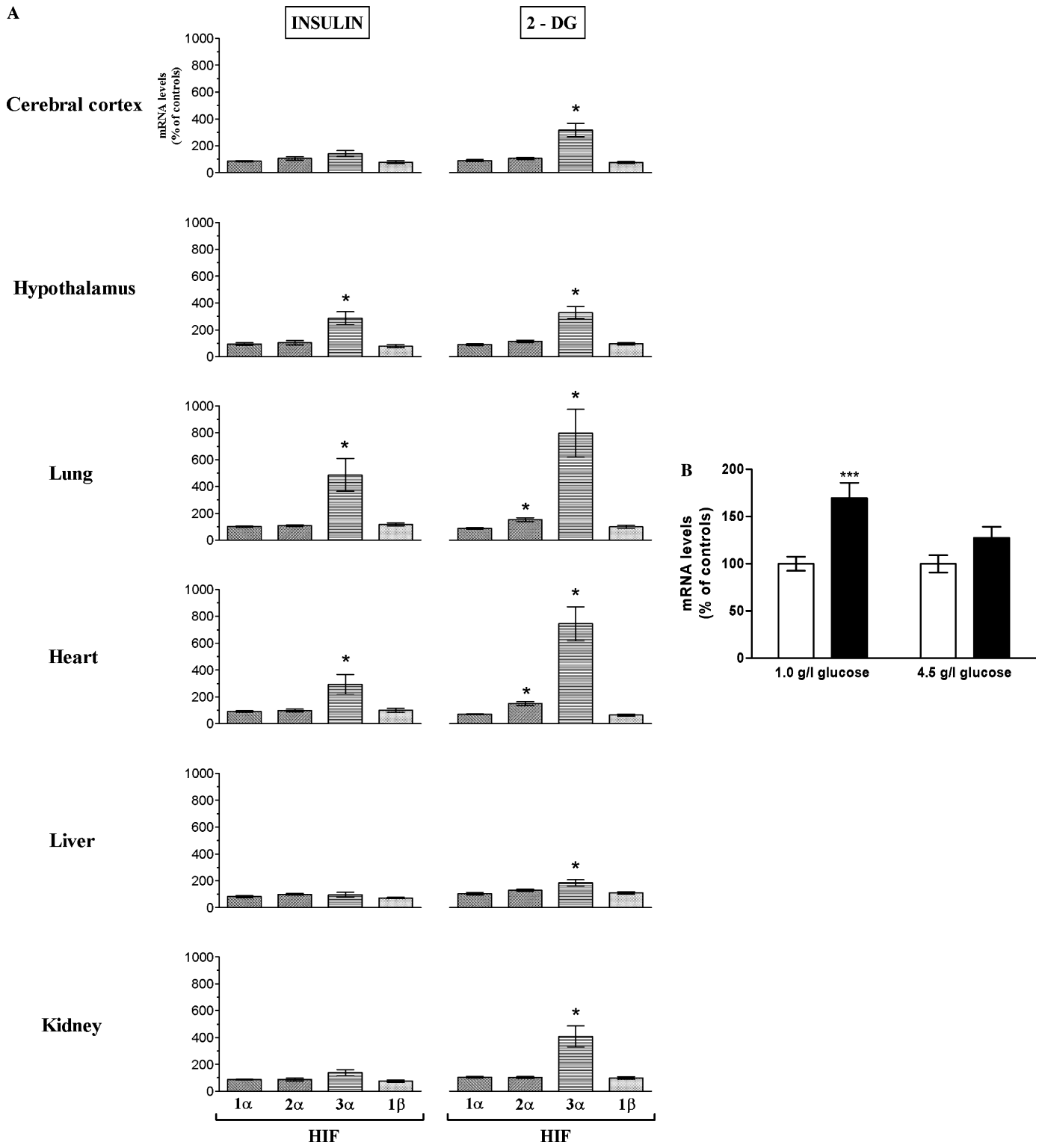

Fig. 2. Effects of metabolic derangements on the gene expression of various HIF subunits. (A) Influence of insulin and 2-deoxy-D-glucose (2-DG) on the mRNA expression of HIF-1α, HIF-2α, HIF-3α, and HIF-1β. Data are presented as means ± SEM. $^{*}P < 0.05$ as compared with experimental groups receiving saline. (B) In HT-22 neuronal cells, 2-DG was applied in culture media together with 1 mg/ml or 4.5 mg/ml glucose, respectively. $^{***}P < 0.01$ as compared with controls.

atom by the substitution of hydrogen for a hydroxyl group, leads to disruption of glucose metabolism by accumulation of the non-metabolisable 2-DG-phosphate (2-DG-PO_4) [13,17]. Additionally, 2-DG for many years is a well-established model for studying derangements in carbohydrate metabolism [18]. A potential influence of 2-DG on the system of HIFs has not yet been determined but the present data indicates that 2-DG is a powerful activator of the

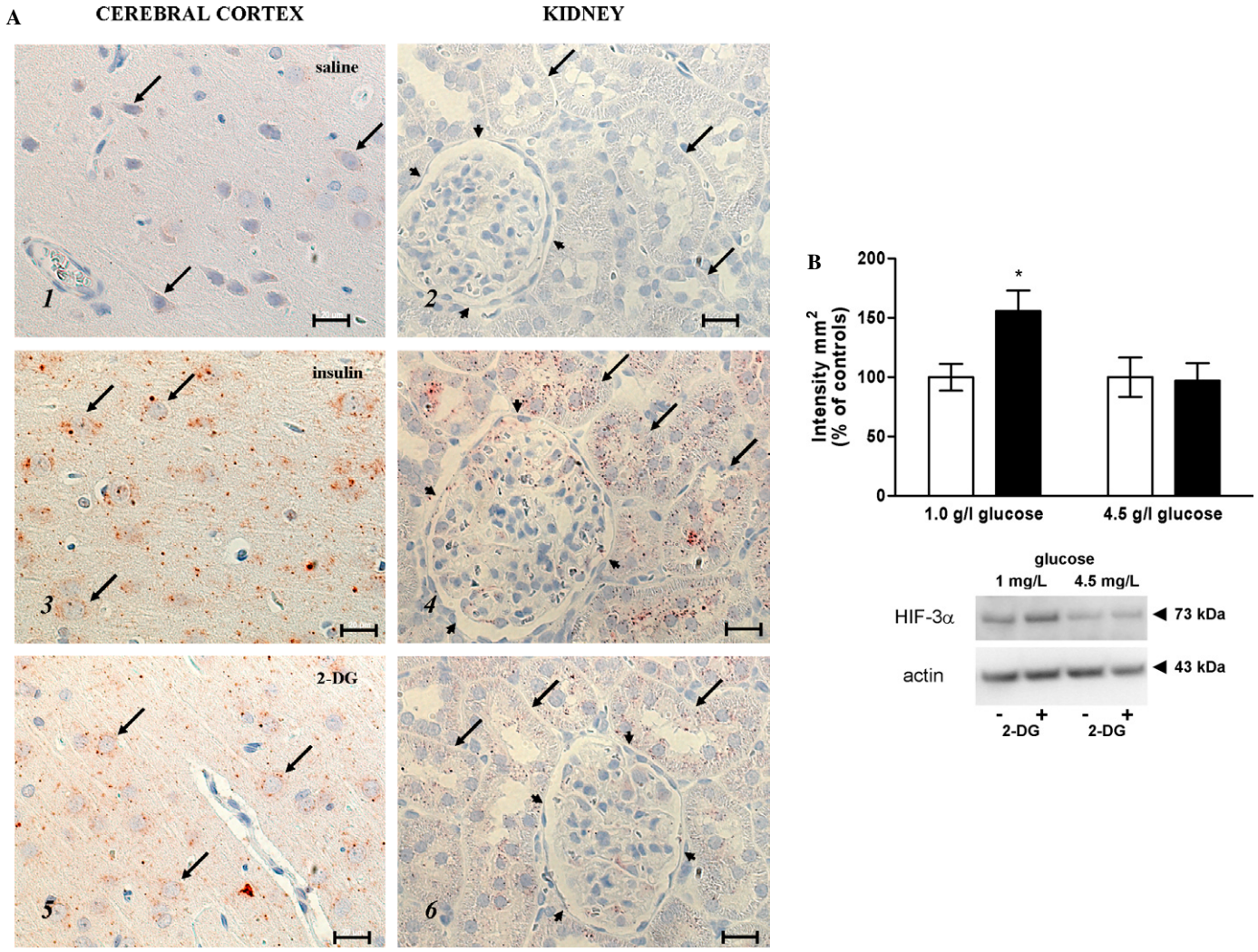

Fig. 3. Effects of metabolic derangements on HIF-3α at levels of protein. (A) Immunohistochemical localisation of HIF-3α in cerebral cortex (1, 3, and 5) and kidney (2, 4, and 6). Wistar rats were treated either with repetitive infusions of saline (1 + 2), insulin (3 + 4) or 2-deoxy-D-glucose (5 + 6). Arrows indicate cortical neuronal cells or renal tubular epithelium, respectively. In the kidney, glomeruli and the surrounding Bowman's capsule are marked with arrowheads (original magnification 400×). (B) Immunoblot analysis of HIF-3α protein in HT-22 cells after incubation with 2-DG in the presence of 1 mg/ml or 4.5 mg/ml glucose, respectively. $^{*}P < 0.05$ as compared with controls.

HIF system. The effect of 2-DG seemed to occur independently from insulin since the measured levels of circulating insulin was not affected by treatment and that again is in line with previous studies [17].

The observed induction of HIF targets, as evidenced by an increase in mRNA amounts of the HIF-dependent target gene GLUT-1 in the hypothalamus, lung, heart, and the liver, suggests a broad activation of the HIF system. Indeed, activation of GLUT-1 by 2-DG was noted in a preceding study but this observation has not been linked towards HIFs [13].

Regarding HIF-1α, levels of mRNA were not influenced by treatment with insulin. This finding is in line with the fact that HIF-1α and HIF-2α are mainly regulated at the protein level and changes of mRNA levels have been observed only after long lasting severe hypoxic episodes [19]. In contrast, a significant increase in mRNA encoding for HIF-3α was seen in all organs analysed after 2-DG treatment. To rule out indirect effects by neuronal or hormonal influences, HT-22 cells were dispensed with 2-DG. Again, application of 2-DG led to a significant rise of HIF-3α mRNA. Expectedly, during treatment with varying glucose concentrations a competitive interaction between glucose and 2-DG was notable. Overall, the pattern of the observed induction of HIF-3α was similar to that observed recently during systemic hypoxia [9]. Surprisingly, 2-DG was sufficient to promote an increase in HIF-2α mRNA levels in samples of lung and heart. Non-hypoxic induction of HIF-2α at the level of mRNA has been noticed so far only in response to treatment with cytokines such as interleukin-1β (IL-1β) [20] or the iron chelator deferoxamine [21]. It may be speculated that in addition to hypoxic stabilisation, other pathways may exist that serve to regulate this subunit. At levels of protein, the induction was paralleled by a marked accumulation of HIF-α proteins confirming the activation. Remarkably,

HIF-3α proteins were found to be localised mainly in the perinuclear cytosol in all samples examined since it was observed that HIF-1α or HIF-2α proteins, respectively, preferably accumulate in the nucleus [22]. In the case of 2-DG, a possible explanation could be a recent observation that ATP depletion induced by 2-DG inhibited the Ran-dependent nuclear transport [23]. Taken together, a direct interaction between 2-DG and the HIF system is very likely. This observation in turn might have impact on the treatment of cancers where possible interactions may arise due to the essential role of HIF-1α and HIF-2α in the survival of neoplastic tissues [3].

Application of insulin seemed to induce a less pronounced activation of the HIF system when compared to 2-DG treatment. The cellular cross-talk of insulin and HIF-2α or HIF-3α is not known. Regarding HIF-1α, the mostly favoured signalling pathway is the phosphatidylinositol 3-kinase via which insulin activates HIF-1 [1,17]. This activation seems to occur as a result of protein modification since it was recently reported that hypoxia but not insulin is sufficient to stabilise HIF-1α at levels of protein [24]. Notably, both insulin- and hypoxia-induced target gene expression seems to require the HIF-1β subunit [24]. It was therefore postulated that another as yet unidentified dimerisation partner of HIF-1β exists that is directly activated by insulin. Our study provides evidence that this mechanism is functional *in vivo* and involves additionally HIF-3α. Whereas levels of mRNA encoding for HIF-1α and HIF-2α remained unchanged during hypoglycaemia, insulin was capable of inducing HIF-3α at the transcriptional level, as reflected by increased mRNA amounts in hypothalamic samples, lung and myocardium after termination of the experimental procedure. At the protein level, a marked accumulation of HIF-3α protein was noted. As mentioned above, proteins again were found to be localised outside the nucleus. It may also be speculated that this is a special feature of HIF-3α due to its antagonistic properties concerning HIF-1α, but this must be clarified by future examinations. Sufficient functional activation of the HIF system was further confirmed by the fact that application of insulin produced a significant increase in GLUT-1 mRNA amounts even though it occurred exclusively in the lung. Up to date, a direct activation of GLUT-1 by insulin has not been reported. Therefore, it might be hypothesised that insulin via HIF triggers target genes other than those examined, i.e., genes that may be necessary for an appropriate cellular response to hypoglycaemia.

Taken together, these observations may contribute to the understanding of the pathophysiology of metabolic diseases, and particularly diabetes. A clinical impact could arise from extension of knowledge about HIF-3α and its splice variants, respectively, as a feasible repressor of the HIF system [25] in the potential treatment of diabetic retinopathy. A better knowledge about HIF-3α and its induction by non-hypoxic triggers may assist in the development of therapeutic strategies for preventing retinal neovascularisation.

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