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Mitochondrial succinate is instrumental for HIF1α nuclear translocation in SDHA-mutant fibroblasts under normoxic conditions

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The genes encoding succinate dehydrogenase (SDH) subunits B, C and D, act as tumour suppressors in neuro-endocrine tissues. Tumour formation has been associated with succinate accumulation. In paraganglioma cells, two forms of SDHA (type I, II) were found which might preclude significant succinate accumulation in the case of a mutation in either form. In fibroblasts only SDHA type I is found. In these cells, SDHA type I mutation leads to SDH deficiency, succinate accumulation and hypoxia-inducible factor 1α(HIF1α) nuclear translocation. HIF1α nuclear translocation was not observed in ATPase-deficient fibroblasts with increased superoxide production and was found to be independent of cellular iron availability in SDHA-mutant cells. This suggests that neither superoxides nor iron were causative of HIF1α nuclear translocation. Conversely, α-ketoglutarate (α-KG) inhibits this nuclear translocation. Therefore, the pseudo-hypoxia pathway in SDH-deficient cells depends on the HIF1αprolyl hydroxylase product/substrate (succinate/α-KG) equilibrium. In SDH deficiency, organic acids thus appear instrumental in the HIF1α-dependent cascade suggesting a direct link between SDH and tumourigenesis.

INTRODUCTION

Inherited respiratory chain (RC) deficiencies represent a heterogeneous group of diseases in both their clinical presentation and their molecular basis (1,2). Defective succinate dehydrogenase (SDH) activity causes early onset encephalopathy (3) or tumour of the paraganglias, i.e. paragangliomas (PGs) and/or pheochromocytomas (4). So far, the former clinical presentation has been associated with mutations in the gene encoding the SDHA-subunit only, while mutations in the SDHB, C and D subunits result in tumour formation (5). Tumour formation requires cell proliferation in conditions of restricted oxygen availability, and activation of the hypoxic/angiogenic pathway (6). This involves the concerted action of a series of proteins whose transcription relies on hypoxia-inducible factor 1α (HIF1α) protein nuclear translocation (HIF-NT) (7). Accordingly, an increased expression of the hypoxia-inducible transcription factors, HIF1α and EPAS1/HIF2α(endothelial PAS domain protein), and one of their target genes, vascular endothelial growth factor has been demonstrated in PGs (8).

As a possible mechanism for HIF-NT, we and others have suggested the inhibition of the prolyl hydroxylase (PHD)-catalyzed HIF1α-prolyl hydroxylation (first step in

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proteasome-catalyzed HIF1α degradation) (9,10). Such an inhibition can result from decreased oxygen tension (11), superoxides (12), iron chelation (13), iron oxidation (14), or *in vitro* by product inhibition, i.e. succinate (M. Selak and E. Gottlieb, Euromit 6, Nijmegen, July 1–4, 2004). There are now several lines of evidence suggesting that succinate accumulation plays a key role in HIF-NT in tumours. However, the pseudo-hypoxia occurring in tumours makes it difficult to ascertain the primary role of succinate accumulation in triggering the hypoxia-sensitive pathway.

HIF-NT linked to SDH deficiency should result from SDHA mutations. We, therefore, studied the HIF-NT under controlled normoxia condition in a cell model of an inherited SDH-deficiency resulting from a mutation in SDHA.

RESULTS

Succinate accumulates in SDHA-mutant fibroblasts

Organic acid analysis of the culture media from subconfluent fibroblasts revealed a significant accumulation of succinate in SDHA-mutant fibroblasts (Fig. 1A). Fumarate was hardly detectable, while malate represented <10% of the succinate (Fig. 1A; inset). This organic acid profile was reminiscent of that observed in tumours resulting from a mutation in SDHB (10), but not from a mutation in the RET proto-oncogene (data not shown). Notably, attempts to mimic HIF-NT resulting from SDHA deficiency by providing exogenous succinate to control fibroblasts were unsuccessful, presumably due to the rapid use of cytosolic succinate by functional mitochondria compared with the slow entering of this compound into the fibroblasts. Incidentally, growing SDHA-mutant cells at confluence (>6 weeks, under normoxic conditions) resulted in the appearance of macroscopically visible buds (about 1 per 5 cm^2) (Fig. 1B). These cells were smaller and had fewer outgrowths than adherent cells in the vicinity (Fig. 1B, inset). A modified cell motility due to SDH-deficiency was suggested by a standard wound assay (15) (data not shown).

Two SDHA types are expressed in PGs

Succinate accumulation has been suggested to be a key factor in tumour formation resulting from mutations in the genes encoding SDH subunits B, C and D (9,10,16). A similar succinate accumulation should arise in the case of a deleterious mutation in the SDHA gene. We, therefore, investigated the potential expression in the neuroendocrine tissue of the two SDHA types I and II recently identified in human (17). We found that the two types were easily discriminated by restriction analysis and are concurrently expressed in PGs (Fig. 2A and B). Sequencing cDNA confirmed the presence of SDHA types I and II in PGs (Fig. 2C). The presence of the two SDHA forms in PGs precludes a sufficient succinate accumulation in the neuro-endocrine tissue in the case of a SDHA mutation.

As previously reported (17), an exclusive expression of SDHA type II was found in the Burkitt's lymphoma cell line (Raji cells; Fig. 2A, lane 2 and D). Yet, another malignant cell line, human cervical carcinoma cells (HeLa), only harbours the SDHA type I (Fig. 2A, lane 3). Similarly, we found only SDHA type I in cultured fibroblasts (Fig. 2A, lane 4 and E). We, therefore, decided to study potential HIF-NT in SDHA-mutant.

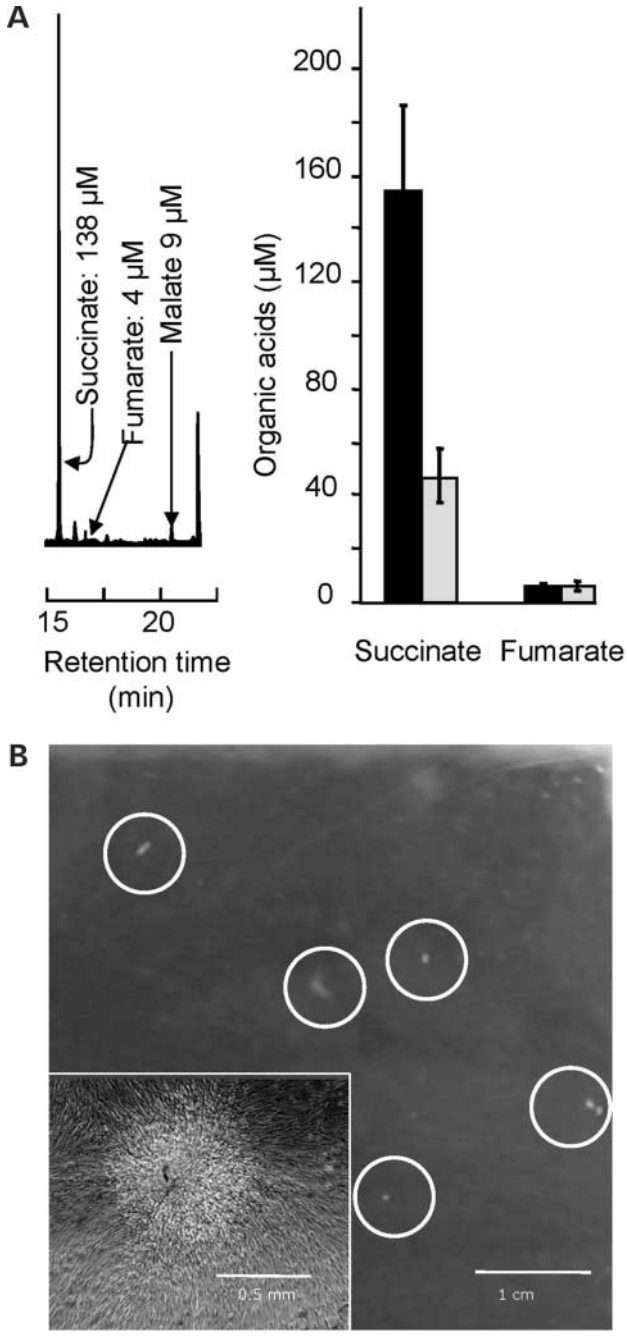

Figure 1. Organic acid accumulation and bud formation in SDHA-mutant fibroblasts. **(A)** Quantification of organic acids in control (grey columns) and SDHA-mutant fibroblast (dark columns) culture media. Measurements were carried out 4 days after medium renewal on subconfluent cultures. Values are mean ± 1 SD ($n = 3$). **(B)** SDHA-mutant fibroblast culture showing macroscopically detectable white spots after 6 weeks in culture; inset: detailed view of one spot.

HIF1α translocates to nuclei in SDHA-mutant fibroblasts

We compared the status of HIF1α protein in control, control plus Co^{2+} and SDHA-mutant fibroblasts. Co^{2+} fully inhibits

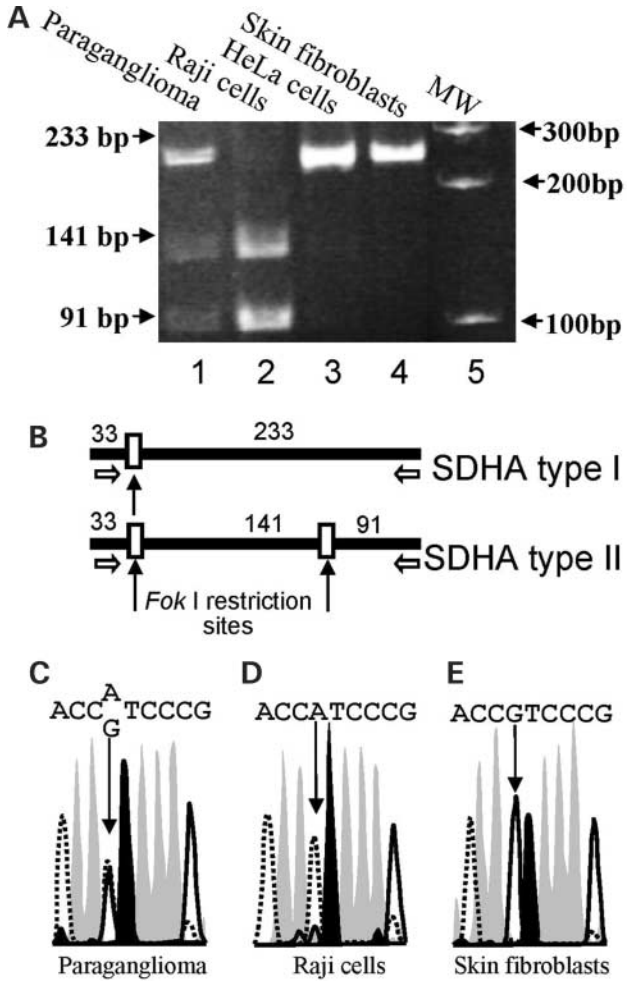

Figure 2. Evidence for two types of SDHA in neuroendocrine tissue. RT–PCR/restriction analysis (**A**) showing the *Fok* I-restriction fragments (**B**) obtained using cDNAs prepared from a PG homogenate, Raji and HeLa cells and control fibroblasts. Sequence analysis of the cDNAs from the previous PG (**C**), Raji cells (**D**) and fibroblasts (**E**) showing the nucleotide composition in each tissue, encoding amino acid 657 of the mature protein either as an isoleucine residue (ATC; type II), a valine (GTC; type I) or both in PG with two types of SDHA.

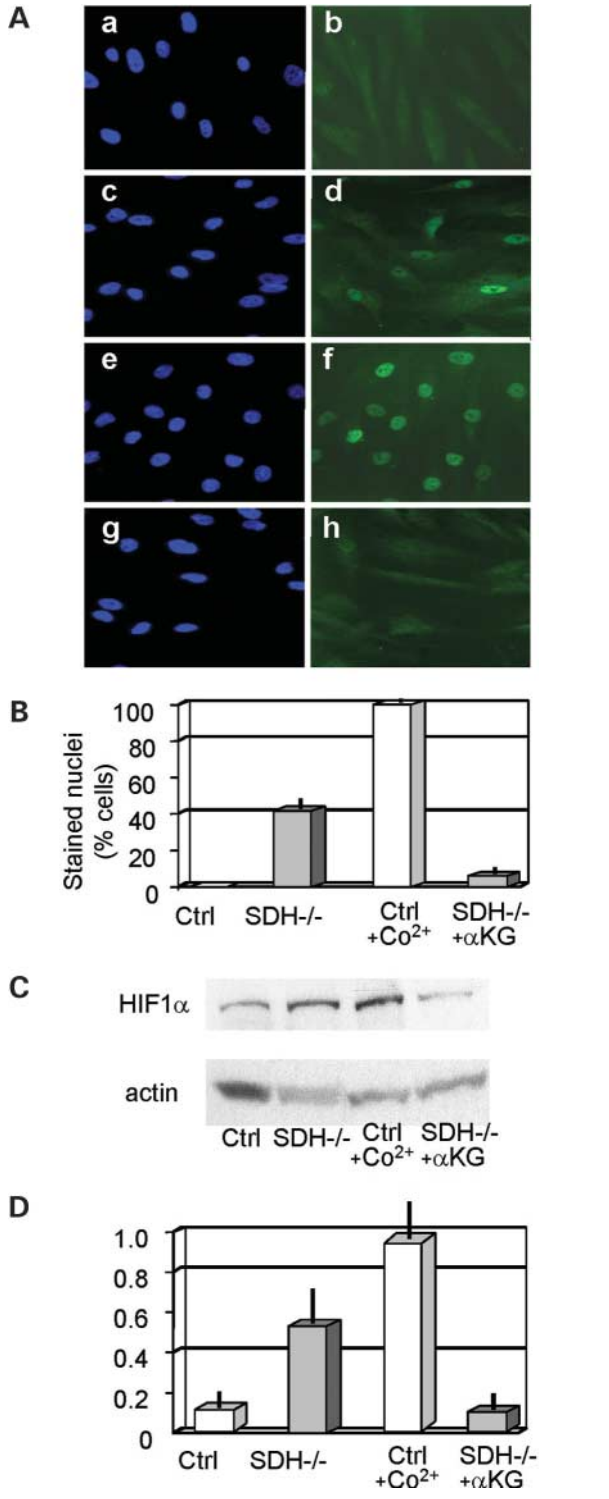

Figure 3. HIF1α nuclear translocation in SDHA-mutant fibroblasts. **(A)** Control fibroblasts (minus or plus cobalt; 12 h, 500 μM; a–b, e–f) and SDHA-mutant fibroblasts (SDHA A554W/A554W; minus or plus α-KG; c–d, g–h) were marked with DNA-staining DAPI (a, c, e, g) or anti-HIF1α antibody (b, d, f, h) as described under Materials and Methods (objective ×20). (**B**) Quantification of anti-HIF1α antibody fluorescence measured on more than 50 cells distributed on four different slides. Numbers are per cent of total cell number, expressed as mean values ±1 SD. (**C**) Total cell extracts (50 μg) were analysed by immunoblotting with antibodies to HIF1α and actin. (**D**) Quantification by densitometry of HIF1α relative to actin using Sigmagel® v1.0.

PHD-catalyzed HIF1α degradation due to iron oxidation and provides a positive control (14). HIF1α was hardly detectable in the nucleus of control cells (Fig. 3Ab and B), but was increased in the nuclei following a 6 h treatment with 0.5 mM Co^{2+} (Fig. 3Af and B). The labelling of HIF1α in the SDH-deficient fibroblasts indicated a nuclear translocation in 40–50% of the cells (Fig. 3Ad and B). Nuclear translocation of HIF1α in SDHA-mutant cells was less than that observed in Co^{2+}-treated control cells (Fig. 3B). Western blot analyses fully confirmed HIF1α accumulation triggered by Co^{2+} in controls and a significant increase in SDHA-mutant cells (Fig. 3C and D).

Superoxides are not sufficient for HIF-NT in fibroblasts

HIF1α may be stabilized and further translocated to the nucleus through PHD inhibition by superoxides. We,

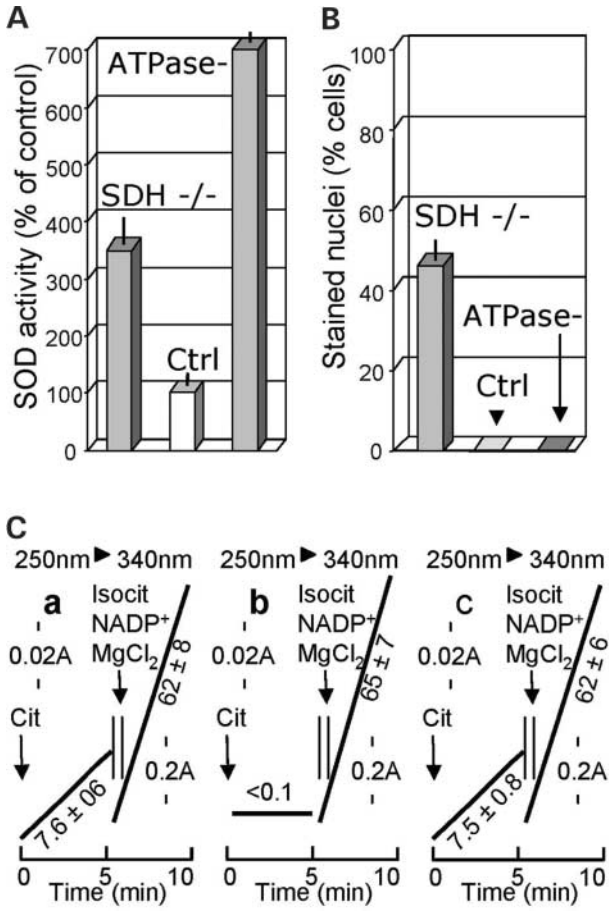

Figure 4. Ruling out superoxide overproduction and change in free iron as primary factors in HIF-NT in SDHA-mutant fibroblasts. **(A)** Total SOD (Mn- and CuZn-dependent) activity measured in SDH-deficient (SDHA A554W/A554W), control and ATPase-deficient fibroblasts. Values are mean ± 1 SD ($n = 5$). **(B)** HIF1α to DAPI nuclei stained ratio in SDH-deficient and ATPase-deficient fibroblasts. Values are mean ± 1 SD ($n = 3$). **(C)** Aconitase and IDH measured in control (a), control incubated 24 h with 150 μM desferioxamine (b) and SDH-deficient fibroblasts (c). Numbers along the traces are nmol/min/mg protein. Cit, citrate; Isocit, isocitrate.

therefore, investigated superoxide steady state and superoxide-induced superoxide dismutase (SOD) activity in SDHA-mutant and ATPase-deficient fibroblasts. While neither cell types showed a change in reduction of the superoxide probe DHFC (data not shown), they both display a significant induction of SOD activity (Fig. 4A), indicative of increased superoxide production. However, no HIF-NT could be observed in ATPase-deficient fibroblasts (Fig. 4B). Mitochondrial superoxide production *per se* appears insufficient to trigger HIF-NT.

Free iron chelation does not induce HIF-NT in SDHA-mutant fibroblasts

Succinate build-up in the cell might affect the iron-dependent PHD reaction by chelating iron (log Kd_{Fe} succinate: 7.49). We measured metabolically available cytosolic iron in control and SDHA-mutant fibroblasts by quantifying aconitase activity, the natural iron sensor present in mammalian cells (18). This enzyme is converted to its non-active iron responsive protein form in case of free iron depletion, as shown by the absence of activity measurable in fibroblasts grown for 24 h in the presence of 150 μM desferioxamine (Fig. 4D, trace b). Aconitase activity and its ratio to isocitrate dehydrogenase (IDH) activity were similar in SDHA-mutant and control cells (Fig. 4D, traces a and c), indicative of normal cytosolic iron in SDHA-mutant fibroblasts. Accordingly, providing reduced iron–transferrin complex to SDHA-mutant fibroblasts did not prevent HIF-NT (data not shown). This ruled out free iron as being instrumental for HIF-NT in SDHA-mutant fibroblasts.

α-ketoglutarate prevents HIF-NT in SDHA-mutant fibroblasts

In order to establish whether HIF-NT results from product/substrate equilibrium of the PHD reaction, we attempted to counterbalance potential succinate (product) effect by providing exogenous α-KG, the PHD substrate. In order to avoid PHD inhibition by iron chelation, we provided α-KG (2.5 mM) concurrently with iron–transferrin. We also verified that α-KG did not reduce the level of SOD activity in SDHA-mutant fibroblasts (data not shown). In the presence α-KG, HIF-NT was no longer observed (Fig. 3Ah and B). Western blot analyses confirmed this result (Fig. 3C and D). Altogether, these data denote that the product/substrate equilibrium of PHD is the controlling factor of HIF-NT.

DISCUSSION

Originally, a subset of hereditary PGs were associated with mutations in the genes encoding SDH-anchoring subunits D and C (4,19). This was taken as an indication that sparing the catalytic core of the enzyme (SDHA–B), whose activity might generate superoxides, was required to trigger tumour formation. This view was re-inforced by the study of *Caenorhabditis elegans* mutant, mev-1 (kn 1), defective in cytochrome *b* large subunit (Cyt-1/ce SDHC) (20). This mutation caused shortened lifespan of the mutant worm in an oxygen-dependent fashion (21). Oxidative stress was demonstrated in the worm and claimed to result from preserved redox activity of the SDHA–B subcomplex (20). This hypothesis became less attractive in the absence of detectable residual succinate-dependent redox activities from the SDHA–B subcomplex in PG samples (8), and, even less, with the report that SDHB mutations cause familial PGs as well (22).

The discovery that a mutation in fumarase can trigger cutaneous and uterine leiomyomatosis and renal cancer (23) suggested that blocking the metabolic step involving SDH and fumarase is crucial for tumourigenesis (24). SDHA mutations, not known to induce tumour formation, should hamper this step as well. The expression of the two SDHA forms in PGs, established in our study, presumably prohibits succinate accumulation in this tissue in the case of a SDHA mutation.

Impairing SDH activity can result in a number of consequences, possibly shared with other types of mitochondrial deficiencies (16). We have attempted to determine which, among the predictably specific consequences of SDH deficiency, could actually be involved in tumour formation. Superoxide overproduction, because it could arise from SDH deficiency and thus trigger tumour formation, represents an obvious candidate (25). Considering the suggested, yet disputable, role of HIF-NT as the primary step of tumourigenesis in neuro-endocrine tissues (26,27), possibly affecting the apoptotic process (28), we decided to study possible HIF-NT in SDHA-mutant cells and to attempt to delineate the underlying mechanism.

We first established that RC-produced superoxides are not *per se* sufficient to trigger HIF-NT. This was observed in SDHA-mutant cells, but not in superoxide-overproducing ATPase-deficient cells. In addition, α-KG, although strongly decreasing HIF-NT, did not change SOD activity in SDHA-mutant fibroblasts. As iron redox status and/or availability could also affect HIF-NT (14), we determined whether adding reduced iron–transferrin complex would oppose HIF-NT in SDHA-mutant fibroblasts. We observed no effect, denoting that reduced iron was not limiting in SDHA-mutant fibroblasts. Accordingly, aconitase activity, used as a sensor for cytosolic free iron, was not significantly altered in SDHA-mutant as compared with control fibroblasts. Together, these experiments indicate that neither iron redox status nor iron availability plays a role in HIF-NT observed in SDHA-mutant fibroblasts.The last parameter that could affect PHD activity is its product/substrate equilibrium (9,10). We found a huge succinate accumulation in SDHA-mutant cells as previously detected in PG samples (10,16). The amounts were in the ranges reported to inhibit the *in vitro* PHD reaction (9), providing a simple explanation for the HIF-NT. The counterbalancing effect of α-KG addition on HIF-NT was used to investigate this hypothesis. Our study shows the spectacular effect of α-KG on HIF-NT, indicative of the crucial role of substrate/product equilibrium in controlling PHD *in situ*.

Altogether, our results support the link between HIF-NT and succinate accumulation, reversible by α-ketoglutarate **(**α-KG**)**. This suggests a crucial role of tricarboxylic acid cycle intermediates in controlling the pseudo-hypoxic pathway, even under normoxic conditions, possibly opening a way for new therapeutic approaches.

MATERIALS AND METHODS

Patients

Patient 1 harboured a deleterious homozygous mutation in the coding sequence of the SDH flavoprotein subunit A (a C to T transition at nt 1684, changing a highly conserved arginine to tryptophan; Arg554Trp) (3). The resulting SDH deficiency (<20% residual activity) caused a severe leukodystrophy with Leigh's syndrome in the two siblings from this consanguineous family. Patient 2 presented a Leigh syndrome caused by a severe deficiency in mitochondrial ATPase resulting from a mutation in the mitochondrial ATPase 6 gene (T8993G; >95% mutant mitochondrial DNA) (29). Patient 3 tumoural tissues (PGs) were obtained following surgery and immediately frozen dry in liquid nitrogen until required for RNA extraction and enzyme studies. A genetic test was performed to search for hereditary PG or familial pheochromocytoma (analysis of SDHD, SDHB, SDHC, RET and VHL genes) on leukocyte DNA of the patient (30). Tumour used in this study harboured a deleterious mutation in the RET proto-oncogene. A written informed consent was obtained in accordance with the national ethical rules.

Cell culture

Fibroblasts were derived from skin biopsies from either voluntary controls or the two patients with a RC deficiency. Fibroblasts, Raji and HeLa cells were aerobically grown under standard condition (20% O_2, 5% CO_2) in RPMI 1640 supplemented with 10% foetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, 2.5 μg/ml fungizone, 2.5 mM pyruvate and 200 μM uridine. For enzyme measurements, subconfluent cells were harvested 24 h after medium change. Abnormal cell proliferation was assessed by maintaining confluent cells under normoxic conditions (20%) for at least 6 weeks with a medium change twice a week.

Gas chromatography/mass spectrometry

Organic acids were quantified in tumour homogenates and skin fibroblast culture media after acidification and ethylacetate extraction using gas chromatography/mass spectrometry according to a standard procedure (31).

Enzyme studies

Enzyme measurements were performed on skin fibroblast pellets or PG tissue homogenates. Cells/organelles were disrupted by freeze-thawing. Malonate-sensitive succinate cytochrome *c* reductase (EC 1.3.99.1) and subsequent antimycin-sensitive decylubiquinol cytochrome *c* reductase (EC 1.10.2.2) assays were performed in 10 mM phosphate buffer (pH 7.8), bovine serum albumin (1 mg/ml), 25 μM cytochrome *c*, 200 μM KCN (32). Total SOD activity (EC 1.15.1.1) was spectrophotometrically measured by following sample-induced decrease of pyrogallol oxidation at 420 nm. Aconitase (EC 4.2.1.3) and IDH (EC 1.1.1.42) activities were measured on the same fibroblast sample in 1 ml of 0.1 M Tris–HCl (pH 7.3) in the presence of 0.1% Triton X100. Measurements were taken at 250 nm (detecting aconitate production from 25 mM citrate during aconitase assay) and at 340 nm (allowing to record $NADP^+$ reduction by IDH, in the presence of 5 mM $MgCl_2$, 25 mM isocitrate and 0.8 mM $NADP^+$) during the time course of the experiment. All enzyme activities were measured at 37°C. Protein concentration was determined by the Bradford assay. Reagents were of the highest grade commercially available from Sigma-Aldrich Company (France).

Molecular analyses

Total RNA from PGs, skin fibroblasts, HeLa and Raji cells were extracted and treated by DNase I using the RNeasy Kit (Qiagen GmbH, Hilden). RNA was reverse transcribed using the GeneAmp® RNA PCR core kit (Applied Biosystems,

England). PCR was performed on 4 μl of cDNA in a 50 μl amplification mixture containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1.2 mM dNTPs, 0.5 μM each primers (forward; 5′-GTGCGGATTGATGAGTACGATT-3′; reverse: 5′-CACATGCATGAGCTATTATACATAA-3′). After 35 cycles (96°C, 30 s; 60°C, 30 s; 72°C, 30 s), amplification products (266 kb) were either directly sequenced by using the fluorescent dideoxyterminator method on an ABIprism 3100 automatic sequencer (Applied Biosystems, England) or digested (20 μl) 2 h at 37°C with *Fok* I restriction enzyme, followed by separation on a 6% acrylamide gel for 1.5 h at 200 V.

Immunofluorescence microscopy

Cells were grown in eight-well chamber slides to 60–70% confluence to avoid hypoxia, fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 1% Triton X-100 in phosphate buffered saline solution (PBS) for 15 min. Cells were then incubated for 30 min at room temperature with 10% normal sheep serum (Dako Corp., CA, USA) in PBS and for 2 h with an anti-HIF1α monoclonal antibody (Becton and Dickinson, Biosciences, NJ, USA) at the dilution of 1:100 in PBS containing 5% sheep serum. Following three washes with PBS for 15 min with agitation, cells were fluorescently labelled for 1 h with an AlexA Fluor® A488-conjugated goat anti-mouse antibody (Molecular Probes, France) at the dilution of 1:1000 in PBS containing 5% sheep serum and washed three times for 5 min with PBS. Nuclei were stained with diamino phenylindol (DAPI; 1 μg/μl for 2 min). Epifluorescence microscopy was performed using an inverted microscope (Eclipse TE 300, Nikon, France). Images were captured with a chilled CCD camera (Ropper Scientific, NJ, USA) and analysed with the Meta-View Software (V.4.6).

Western blot analyses

Cells were lysed in a RIPA buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40; 0.25% Sodium deoxycholate, 1 mM PMSF, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 10 μg/ml Pepstatin). The protein concentration was determined using the Bradford assay and 50 μg of total cellular extracts were separated by SDS–PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., MA, USA). HIF1α and β-actin (Chemicon, Temecula, CA, USA) were revealed with specific antibodies. The immunoreactive bands were revealed using the ECL system (Amersham Pharma Biotech). Protein concentration was determined by densitometry using sigmagel® v1.0 (Jandel Scientific).

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Conflict of Interest statement. No conflict of interest.

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