Bafilomycin A1 activates HIF-dependent signalling in human colon cancer cells via mitochondrial uncoupling

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INTRODUCTION

Pleomacrolide Baf (bafilomycin A1) [1], known as a potent inhibitor of the V-ATPase (vacuolar H\(^+\)-ATPase) [2–4], regulates a number of metabolic processes associated with translocation of protons into the acidic compartments. Treatment with Baf causes lysosome dysfunction, decrease in pH and elevation of cytosolic Ca\(^{2+}\), and neurotransmission failure in neural cells [5–10], by increasing Ca\(^{2+}\) permeability transition pore opening [11] and cell death. Impaired fusion of autophagosomes with dysfunctional lysosomes inhibits autophagy [12] and triggers apoptosis [13] and therefore is considered to be one of the main mechanisms of the anticancer effect of Baf. In agreement with pro-apoptotic properties, Baf and another V-ATPase inhibitor CMA (concanamycin A) were also shown to induce mitochondrial depolarization and apoptosis in leukaemic monocytes by activating the production of nitric oxide [14].

Anti-cancer effects of Baf connect V-ATPase and HIF-1 (hypoxia inducible factor 1) [15–17]. Baf was shown to promote direct interaction of V0 subunit c (ATP6V0C) with HIF-1\(\alpha\) and its stabilization, which, in turn, induced p21\(^{WAF1/CIP1}\), mediated growth arrest in a number of cancer cell lines [15–17]. ATP6V0C protects HIF-1\(\alpha\) from interaction with VHL (von Hippel–Lindau) E3 ubiquitin ligase and subsequent proteasomal degradation. In normoxia HIF-1\(\alpha\) subunits are maintained at low levels. Upon hydroxylation by O\(_2\)-dependent prolyl and asparaginyl hydroxylases [PHD (prolyl hydroxylase domain) 1–4 and...
FIH (factor inhibiting HIF), HIF-α subunits undergo degradation through VHL protein or lose their DNA binding capacity [18]. The rate of HIF-α degradation decreases at low O2 (hypoxia) and α-ketoglutarate availability, or upon pharmacological inhibition of PHDs [19].

Identifying new molecules targeting HIFs is of high practical interest, because HIFs regulate cancer cell metabolism and tumour progression [20]. The majority of therapeutic compounds targeting cancer via HIF signalling have an inhibitory effect on HIF stabilization/transactivation (see [21]). However, the results on Baf-dependent cancer suppression mediated by HIF-1 contradict the role of HIF-1α as a transcriptional regulator of cancer cell survival and progression of many tumours [20], including colon cancer [22–24]. In solid tumours, deep deoxygenation [25] contributes to HIF-1α stabilization, the latter promotes cancer development through VEGF (vascular endothelial growth factor)-dependent vascularization and pro-glycolytic rearrangement of cell metabolism [20]. Surprisingly, expression of common HIF-1 targeted genes was not affected by Baf [17].

Despite the remarkable effect of Baf on HIF-1α, stabilization of HIF-2α and its role in Baf-dependent cancer suppression has not been analysed. Yet, the unique transcriptional signatures of HIF-1 and HIF-2 make them very specific regulators of tumour progression. Thus, HIF-2 is associated with malignant tumours and poor survival prognosis [26–29], while accumulation of HIF-2α in a number of HIF-2-associated tumours was linked to decreased patient mortality [30,31].

The recently found uncoupling effect of Baf on isolated mitochondria [32] and neuronal cells [33] may also contribute to stabilization of HIF-α subunits by activating respiration and decreasing iO2 (intracellular O2) levels. Indeed, it is known that increased mitochondrial activity regulates HIF-α stabilization in an iO2-dependent manner [34–37]. Knowing that relative oxygenation of respiring cells decreases at reduced O2 availability [38], we hypothesized that at physiological O2 levels (2–12% O2), treatment with Baf can shift cell deoxygenation to levels sufficient for HIF-1α and HIF-2α stabilization. To check this, we compared the effect of Baf on HIF signalling in two human colon cancer cell lines, exposed to 6% O2 for 5 h. As models we used actively respiring WT (wild-type) HCT116 cells and low respiring HCT116 cells deficient in SCO2 protein (synthesis of COX 2, which is involved in the COX (cytochrome c oxidase) assembly [39]).

**MATERIALS AND METHODS**

**Materials**

iO2 sensitive probe NanO2 [40] was from Luxcel Biosciences. OptiMEM I medium, TMRM (tetramethyl rhodamine methyl ester), MTG (Mito Tracker® Green) FM, DAPI (4,6-diamidino-2-phenylindole), Fluo-4 AM and ProLong® Gold were from Invitrogen Life Technologies. Amersham ECL™ Prime Western blotting reagent was from GE Healthcare Life Sciences; pre-made acrylamide gels, running and transfer buffer were from GenScript; BCA™ Protein Assay kit was from Thermo Fisher Scientific; protease inhibitor mixture tablets were from Roche Applied Sciences; CellTiter-Glo® ATP Assay, ImProme-II™ RT (reverse transcription) System and PCR Master Mix were from Promega. SafeView DNA stain was from NBS Biologicals. Primary antibodies were against: α-tubulin (T5168, Sigma), HIF-1α and HIF-2α (MAB1536 and AF2886, R&D Systems), HIF-1α (NB100-105, Novus Biologicals), HIF-1β (ab2771, Abcam), HIF PHD2 (4835, Cell Signalling Technology), HRP (horseradish peroxidase)-conjugated mouse anti-goat, mouse anti-rabbit and donkey anti-mouse IgG (A9452, A1949 and A0168, Sigma). Baf, CMA, DMOG (dimethyl-oxalylglycine), DEPC (diethyl pyrocarbonate), collagen IV, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and other reagents were from Sigma–Aldrich. Primers were designed using Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Supplementary Table S1 at http://www.bioscierep.org/bsr/032/bsr0320587add.htm) and were synthesized by Sigma–Aldrich. Plasticware was from Sarstedt, Corning Life Sciences (Corning), Ibidi and Greiner Bio One.

**Tissue culture**

Human colon cancer WT (HCT116 WT) cells were obtained from the European and American Collections of Cell Cultures, HCT116 SCO2−/− [41] were kindly provided by Professor P.M. Hwang (Laboratory of Cardiovascular and Cancer Genetics, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A.). Cells were maintained in the McCoy medium supplemented with 10% FBS (fetal bovine serum), 2 mM l-glutamine, 100 units/ml penicillin/100 μg/ml streptomycin (P/S) and 10 mM Hepes, pH 7.2. For iO2 and cellular ATP measurements, WT and SCO2−/− cells were seeded in the same medium at 1×103 and 8×103 cells per well, respectively, on 96-well plates coated with PDL (poly-D-lysine, 0.01 mg/ml) and grown for 16 h prior to the experiments. For immunofluorescence analysis cells were seeded at 1×105 and 8×104 cells per well in Ibidi 12-μl-chambers coated with a mixture of collagen IV (0.007 %) and PDL (0.003 %). For protein and RNA isolation cells were seeded at 1.1×104 (WT) and 8×105 (SCO2−/−) cells per well on 12-well plates (Corning) pre-coated with PDL and grown for 16 h.

**Monitoring of iO2**

For the iO2 assay, cells were loaded with NanO2 probe by incubating them in a regular medium containing 10 μg/ml probe at 37 °C for 18–24 h as described [42]. Measurements were conducted at 37 °C in serum-free McCoy’s medium buffered with 20 mM Hepes (pH 7.2), typically with 48 samples of adherent cells in 200 μl of the air-saturated medium (see Supplementary Figure S1). The cells were treated with Baf and other compounds for 30 min and then the plate was transferred to a TR-F (time-resolved fluorescence) plate reader Victor 2 (PerkinElmer Life Science) placed in a hypoxia chamber (Oxy Scientific) equilibrated at 6% atmospheric O2 (~60 μM dissolved O2). Monitoring was started using 340 nm excitation and 642 nm emission filters.
Uncoupling with bafilomycin activates HIF signalling in cancer cells

Each sample well was measured repetitively every 5–10 min for 6 h by taking two intensity readings at delay times of 30 and 70 μs, using a gate time of 100 μs [43]. The TR-F intensity signals were converted into phosphorescence lifetime (τ) values as follows: \( \tau = (t_1 - t_2) / \ln(F_1/F_2) \), where \( F_1 \) and \( F_2 \) are the intensity signals at delay times \( t_1 \) and \( t_2 \). The latter were related to \( [iO_2] \): \( [iO_2] = 66.72 \times \ln(37.62 / (t_1 - t_2)) \). From the resulting \( [iO_2] \) profiles, the rate of cell deoxygenation was calculated as \( O_2 \) consumed in 1 min by 10⁶ cells (nM/min per 10⁶ cells).

**Measurement of the mitochondrial mass and ΔΨ_m (membrane potential), cytosolic Ca²⁺ and cellular ATP**

Cells were incubated with 50 nM MTG, 20 nM TMRM and 2.5 μM Fluo-4 AM probes in OptiMEM I (supplemented with an additional 1 mM Ca²⁺) for 30 min in CO₂ incubator at 37 °C and then measured. During the measurements, TMRM was maintained in OptiMEM at 20 nM. AM probes were de-esterified for 15 min prior to each experiment.

Mitochondrial mass, ΔΨ_m and cytosolic Ca²⁺ were measured by live cell imaging of MTG, TMRM and Fluo-4 fluorescence, respectively, using an Olympus FV1000 confocal laser scanning microscope with controlled CO₂, humidity and temperature. MTG, and Fluo-4 were excited at 488 nm with emission collected at 500–550 nm. TMRM was excited at 543 nm, emission was collected with a 555–600 nm filter. In all the experiments, fluorescence and DIC (differential interference contrast) images were collected in a sequential mode with a ×60 oil immersion objective in a number of planes using 0.5 μm steps. The resulting z-stacked images were analysed using FV1000 Viewer (Olympus), Adobe Photoshop and Illustrator software.

Total cellular ATP was quantified using CellTiter-Glo® assay following the manufacturer’s protocol. Briefly, the cells were lysed with CellTiterGlo® reagent and after intensive shaking the lysates were transferred to white 96-well plates (Greiner Bio One) and measured on a Victor 2 reader under standard luminescence settings. Conditions of treatment with various effectors are described in the Results section.

**Immunofluorescence**

Immunofluorescence analysis was performed similarly to [44]. Briefly, HCT116 cells were seeded on Ibidi chambers, grown and treated as described above, washed with PBS, fixed with 3.7 % PFA (paraformaldehyde) in PBS, quenched with 50 mM NH₄Cl, permeabilized with 0.25 % TX100, blocked with 5 % BSA, incubated with anti-HIF-1α (Novus Biologicals) primary and fluorescent anti-mouse secondary antibodies conjugated with Alexa Fluor555 dye (Invitrogen), counterstained with DAPI, mounted with ProLong® Gold and air-dried. The intensity and localization of HIF-1α staining was analysed by confocal microscopy (Olympus): DIC, DAPI and Alexa-555 images were collected with a ×60 oil immersion objective in eight planes using a 0.5 μm step in sequential mode using standard excitation and emission wavelengths. The resulting single plane (DIC) and z-stacked fluorescent images were analysed using FV1000 Viewer software (Olympus), Adobe Photoshop and Illustrator.

**Protein isolation for Western blot analysis**

Whole cell lysate proteins were prepared as described in [33]. Briefly, HCT116 cells grown in the McCoy’s medium on 12-well plates and treated as described above, were washed twice with PBS containing phosphatase inhibitors (Active Motif) and lysed on ice with lysis buffer, containing 150 mM NaCl, 1 mM EDTA, 1 % IGEPAL® CA-630, 50 mM Hepes (pH = 7.5) and protease inhibitors. After lysate clarification by centrifugation for 10 min at 14000 g and 4 °C, total protein concentrations were determined using BCA™ Protein Assay kit and normalized. Proteins were denatured in × 1 Laemmli loading buffer at 95 °C and separated by SDS/PAGE in 10 % and 4–20 % gradient acrylamide gels, transferred to a 0.2 μm Immobilon™-P PVDF membrane (Sigma) using a Hoefer™ TE 22 wet mini-transfer system and probed with antibodies listed above. The signals were developed with Amersham ECL™ Prime reagent and visualized on LAS-3000 Imager (Fujifilm) using Image Reader LAS-3000 2.2 software. Quantitative image analysis was performed with ImageJ program using α-tubulin signals for normalization. Images were processed with Picasa, Photoshop and Illustrator programs.

**Analysis of the HIF-1α HRE (hypoxia response element)-binding activity**

Nuclear and cytosolic protein fractions were prepared from the cells using Nuclear Extract Kit (Active Motif). The DNA-binding activity of HIF-1α was analysed using TransAM™ HIF-1 kit (Active Motif) according to the manufacturer’s protocol. Briefly, 5 μg of proteins were applied on the 96-well plate, pre-coated with an oligonucleotide containing a HRE (5′-TACGTGCT-3′) from the EPO (erythropoietin) gene, and incubated for 1 h. With serial washes after each step, primary mouse anti-HIF-1α and secondary anti-mouse HRP-conjugated antibodies were sequentially measured for 1 h. Then signals were developed and absorption was measured on a TECAN plate reader at 450 nm with a reference wavelength of 655 nm.

**Gene expression analysis by RT–PCR**

Total RNA was isolated using a RNA isolation kit (Promega) as per the manufacturer’s protocol. RT reaction was performed using ImProm-II™ RT System (Promega) at 42 °C for 1 h using 2 μg of total denatured DNA-free RNA in 50 μl reaction mixture containing RT buffer, oligo(dT)₅ primers, dNTPs, RNase inhibitor and reverse transcriptase. After heat inactivation of the enzyme, standard PCR was performed on Mastercycler (Eppendorf). PCR was carried out at low number of cycles (25–30) in order to catch a difference in the number of cDNA copies between the samples. Each reaction was performed in triplicate in three independent experiments. PCR products were separated on agarose gels, stained with SafeView probe (NBS Biologicals), visualized using gel documentation system (Hoefer) and analysed using ImageJ program.

**Statistics**

The data were evaluated for statistical difference using two-tailed Student’s t test. A 0.01 level of confidence was accepted as
A. V. Zhdanov, R. I. Dmitriev and D. B. Papkovsky

Figure 1  
Baf increases respiration in HCT116 cells through mitochondrial uncoupling

(A) The O2 profiles in WT and SCO2−/− HCT116 cells treated with 0.25 μM Baf at 6% hypoxia. Cells were loaded with an O2 sensing probe NanO2 and iO2 levels were monitored for 5 h. In control samples respiration was blocked with AA (10 μM). (B) Treatment with Baf partially depolarizes the mitochondrial membrane potential (∆Ψm) in WT cells, as monitored by TMRM staining (20 nM in solution). (C) In SCO2−/− cells the ∆Ψm is reduced, as compared with WT. (D) A reduction in the ∆Ψm in SCO2−/− cells is not associated with a decrease in the mitochondrial size, as follows from the comparative analysis of MTG staining and VDAC1 protein levels. Bar represents 20 μm. Asterisks indicate statistical significance.

RESULTS

Baf induces deep and sustained deoxygenation of WT HCT116 cells at 6% O2

We reported recently that treatment with Baf induces sustained mitochondrial uncoupling and reduces iO2 in neuronal cells [33]. Here, to find a link between mitochondrial uncoupling and O2-dependent signalling, we first analysed the effects of atmospheric O2 and Baf concentrations on the local deoxygenation of WT and SCO2−/− HCT116 cells. Using an iO2 sensing probe NanO2 and TR-F plate reader Victor 2 placed in the hypoxic chamber [43], we found that at 5–8% atmospheric O2 Baf (0.15–0.35 μM) induces profound deoxygenation of WT cells (Supplementary Figure S1 at http://www.bioscirep.org/bsr/032/bsr0320587add.htm). Thus, treatment with 0.25 μM Baf caused rapid reduction in iO2, which remained at 0–10 μM levels for several hours (Figure 1A). Oxygenation of the cells treated with DMSO (mock) or another V-ATPase inhibitor CMA; results not shown) was significantly higher (30–40 μM). The effect of Baf was abolished by inhibition of the mitochondrial Complex III with AA (antimycin A), which brought iO2 concentration close to the atmospheric levels (∼60 μM). This respiratory response to Baf was coupled to a partial depolarization of the mitochondria (∆Ψm), seen as a decrease in TMRM fluorescence (Figure 1B). Similar to PC12 cells [33], treatment with Baf decreased pH and increased Ca2+ in the cytoplasm of WT HCT116 (Supplementary Figure S2 at http://www.bioscirep.org/bsr/032/bsr0320587add.htm). In contrast, for SCO2−/− cells iO2 profiles were similar to those in WT cells treated with AA, regardless of the presence of Baf.
Uncoupling with bafilomycin activates HIF signalling in cancer cells

(Figure 1A). This result was expected, since SCO2−/− cells deficient in the mitochondrial Complex IV [41] exhibit glycolytic type ATP production, low OCR (O2 consumption rate) and high iO2 levels [45].

Live cell imaging and Western blot analysis revealed no difference in the VDAC1 (voltage-dependent anion channel 1) levels and MTG fluorescence, demonstrating that relative mitochondrial mass in WT and SCO2−/− cells was similar. A decrease in TMRM staining indicated partial depolarization of the ΔΨm in SCO2−/− cells (Figures 1C and 1D). These data confirm that the decrease in O2 consumption in COX-deficient cells was attributable to the respiration and iO2 levels. In agreement with this, stabilization of HIF-1α and HIF-2α in SCO2−/− cells was observed only in the presence of DMOG. This demonstrates that synthesis of HIF-1α and HIF-2α in SCO2−/− cells was preserved.

On the other hand, treatment with CMA also caused significant stabilization of HIF-α subunits in WT cells (Figure 2A). This increase was less pronounced than in the samples treated with Baf or DMOG, although it proved that HIF-α can also be stabilized by V-ATPase inhibitors as described [17]. Surprisingly, Baf and CMA had practically no effect on HIF-α

Baf induces HIF-1α and HIF-2α stabilization through mitochondrial uncoupling

Activation of the HIF-dependent pathway is a common response of the cell to the hypoxia. Therefore, we addressed a question whether the marked decrease in iO2 induced by Baf leads to stabilization of HIF-1α and HIF-2α at 6% atmospheric O2. We found that in WT cells treated with Baf for 5 h the levels of both HIF-α isoforms were strongly elevated (Figure 2A), as compared with mock control (DMSO) or low resiping SCO2−/− cells. Inhibition of mitochondrial respiration with AA abolished HIF-α stabilization by Baf. ATP levels in the cells treated with Baf/AA were partially reduced, suggesting that the cells experienced energy stress (Figure 2B) and could decrease de novo synthesis of proteins, including HIF-α subunits. To rule out this side effect, we incubated Baf/AA treated cells with DMOG, which is known to stabilize HIF-α in an O2-independent manner through the competitive inhibition of HIF PHDs. We observed that in the presence of DMOG, HIF-1α and HIF-2α were strongly increased in all WT cells, regardless of AA treatment and ATP levels (Figure 2B). This result indicated that the effects of Baf and AA were attributable to the respiration and iO2 levels. In agreement with this, stabilization of HIF-1α and HIF-2α in SCO2−/− cells was observed only in the presence of DMOG. This demonstrates that synthesis of HIF-1α and HIF-2α in SCO2−/− cells was preserved.

Figure 2 Stabilization of HIF-1α and HIF-2α in HCT116 cells upon Baf treatment (0.25 μM) is associated with mitochondrial uncoupling

(A) Western blot analysis demonstrates significant elevation in HIF-1α and HIF-2α levels in HCT116 WT cells treated with Baf for 5 h at 6% O2. This effect is abolished by inhibiting mitochondrial respiration with AA. In the presence of DMOG, the levels of HIF-1α and HIF-2α stabilization is similar in all samples, including those which are treated with Baf/AA. In the presence of CMA, HIF-1α and HIF-2α levels in WT cells are also increased significantly, yet to a lesser degree than upon Baf treatment. (B) Cellular ATP levels are reduced in WT cells treated with Baf/AA for 5 h at 6% O2, regardless the presence of DMOG. Treatment with Baf in galactose (+) medium causes dramatic decrease in the ATP level. (C) The levels of PHD1, PHD3, VHL and ATP6V0C proteins are similar in WT and SCO2−/− cells, while the content of PHD2 in SCO2−/− cells is significantly lower than in WT control. Asterisks indicate statistically significant difference (*P < 0.01 and **P < 0.001, Student’s t test) between the experimental samples and mock control (DMSO). Hash in (A) shows a significant difference between the effects of Baf and CMA.

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stabilization in SCO2^{−/−} cells. According to [17], Baf promotes competition of ATP6V0C with VHL protein for HIF-1α binding, thus protecting HIF-1α from PHD-dependent degradation. We hypothesized that stabilization of HIFs in the SCO2^{−/−} cells did not occur due to a deficiency in ATP6V0C or an increase in VHL or PHD expression. However, Western blot analysis showed no difference between the two cell lines in the levels of PHD1, PHD3, ATP6V0C and VHL (Figure 2C), while PHD2 expression in SCO2^{−/−} cells was even decreased. Under normoxia (20.9 % atmospheric O2), stabilization of HIF-1α and HIF-2α by Baf or CMA was significantly lower than under anoxia or in the presence of CoCl2 (Supplementary Figure S3 at http://www.bioscirep.org/bsr/032/bsr0320587add.htm), in contrast with the effects observed under 6 % O2. Interestingly, in the cells double-treated with Baf and AA the levels of HIF-1α and HIF-2α were decreased, which was similar to the effect observed under hypoxia. This phenomenon was not associated with any ATP reduction and could be linked to the rate of mitochondrial respiration.

Baf activates HIF-dependent pathways
HIF proteins trigger adaptation of the cell to hypoxia upon translocation to the nucleus where they interact with HRE and modulate transcription of the target genes. Using immunofluorescence analysis, we evaluated the levels of nuclear HIF-1α in WT and SCO2^{−/−} cells, treated with Baf, CMA, DMOG and DMSO for 5 h in normoxic and hypoxic conditions. At 6 % atmospheric O2 we found strong HIF-specific nuclear staining in WT cells treated with Baf and DMOG, while in SCO2^{−/−} cells nuclear HIF-1α accumulation was observed only upon DMOG treatment (Figures 3A–3C). No detectable nuclear staining was observed...
in normoxic samples unless they were incubated with DMOG (results not shown). To examine functional activity of HIF-1, we extracted nuclear proteins from the cells treated as above and performed DNA-binding ELISA analysis using TransAM® HIF-1 kit. We found that the efficiency of HIF-HRE-binding correlated with intensity of nuclear HIF-1α staining (Figures 3C and 3D). This indicates that Baf stabilizes functionally active HIF-1, which should alter the expression of target genes. To check this, we examined the effect of Baf on expression of HIF-dependent genes in hypoxic and normoxic conditions.

At 6% O₂ in WT cells treated with Baf we observed a significant increase in mRNA levels for GLUT1 (glucose transporter 1), CAIX (carbonic anhydrase IX) and PHD2, which were comparable with or higher than those in the DMOG control (Figure 3E). The level of PHD2 protein was also elevated (Figure 3F). Treatment with CMA did not affect transcription of HIF-1 target genes (results not shown). In contrast with DMOG control, treatment with Baf under normoxia moderately increased only GLUT1 mRNA level (Supplementary Table S2 at http://www.bioscirep.org/bst/032/bst0320587add.htm). Expression of HIF-target genes in the resting SCO2−/− cells under normoxia was practically the same as in WT cells. No changes in expression of these genes was observed in SCO2−/− cells upon treatment with Baf, while in the presence of DMOG the levels of mRNA for GLUT1, CAIX and PHD2 was moderately increased; gene expression pattern did not change at 6% O₂.

DISCUSSION

In the present study, we describe an activatory effect of moderate mitochondrial uncoupling on HIF signalling. At moderate hypoxia (6% atmospheric O₂) treatment with the K⁺ ionophore Baf activates mitochondrial respiration in colon cancer HCT116 cells and strongly decreases iO₂ (Figure 1A), which is sufficient for considerable stabilization of HIF-1α and HIF-2α (Figure 2A). These results are in agreement with the previously shown key role of mitochondrial respiration in stabilization of HIF-α subunits [34–37].

Experiments with Baf and mitochondrial inhibitor AA on two cell lines exhibiting very different OCR, demonstrate that HIF-α stabilization is driven by a Baf-dependent increase in mitochondrial respiration and cell deoxygenation rather than by other cellular effects of Baf (Figure 2A). The somewhat surprising inhibitory effect of AA on HIF-α stabilization under normoxia (Supplementary Figure S3) is most probably linked to the mitochondrial activity, and needs further investigation. HIF-α stabilization is not associated exclusively with mitochondrial uncoupling; treatment with CMA partially stabilizes HIF-1α and HIF-2α in WT cells, indicating that the ATP6V0C/VHL-dependent pathway [17] also contributes to HIF-α stabilization in colon cancer cells. To our surprise, this pathway seemingly is not active in SCO2−/− cells (Figure 2A), which exhibit unchanged capacity for HIF-1α and HIF-2α production (Figure 2A, effect of DMOG). Noteworthy, VHL and ATP6V0C levels in SCO2−/− and WT cells do not differ (Figure 2C). Moreover, PHD2 levels in SCO2−/− cells are decreased, while PHD1 and PHD3 remain unchanged, suggesting a potentially lower rate of HIF-α hydroxylation and degradation.

In WT HCT116 cells treated with Baf under moderate hypoxia the levels of mRNA for GLUT1, PHD2 and CAIX are significantly increased (Figure 3E), indicating activation of the glycolytic proliferative pathway, which disagrees with previous work [15] in which no significant increase in mRNAs for HIF-1 target genes upon Baf treatment was seen, though their transcription was up-regulated by hypoxia. However, this mismatch has a simple explanation (Figure 4). We performed our experiments at moderate hypoxia (6% O₂), and the Baf-dependent decrease in iO₂ (by 30–40 μM, as compared with mock control, Figure 1A) was sufficient for triggering specific gene expression. In the experimental conditions described in [15], HIF signalling could be inhibited at high atmospheric O₂ by FIH asparaginyl hydroxylase, while under deep hypoxia (1% O₂) HIF-specific gene expression was already up-regulated and treatment with Baf could not increase it any further. In agreement with that, in spite of HIF-α stabilization in the presence of Baf under normoxia, we observed no significant elevation of HIF-target genes, except for a moderate increase in GLUT1 expression (Supplementary Table S2). The latter, however, most probably was HIF-independent and resulted from general activation of glycolysis upon mitochondrial uncoupling by Baf.

Recently it was shown that Baf treatment of PC12 cells is coupled with a dramatic elevation of free cytosolic Ca²⁺ due to Ca²⁺ release from acidic compartments and partially from the mitochondria [33]. In hypoxia conditions, cytosolic Ca²⁺ overload not only increases the risk of energy crisis, but can also regulate HIF signalling on transcription, translation and degradation levels (for review see [46]). In the present study, we observed substantial increases in cytosolic Ca²⁺ in HCT116 cells treated with Baf (Supplementary Figure S2). However, we did not evaluate the putative mediatory role of Ca²⁺ in Baf-dependent
stabilization of HIF-1α and HIF-2α, because inhibition of cellular Ca²⁺ fluxes decreases respiratory response to Baf and affects O₂ levels [33].

Mitochondrial uncoupling is implicated in the cell-specific toxicity of many drugs [47]. This emphasizes the potential importance of the novel Baf-specific HIF-targeting mechanism for cancer therapy. Moreover, our results indicate that mitochondrial UCPs (uncoupling proteins), which are often expressed in tumors [48–50], may also be involved in HIF-specific regulation of cancer cell metabolism. Similar to pharmacological uncouplers, UCPs accelerate electron flow along the ETC (electron transport chain), thus elevating respiration. Uncoupling increases local O₂ mours [48–50], may also be involved in HIF-specific regulation of cancer therapy. Moreover, our results indicate that mitochondrial toxicity of many drugs [47]. This emphasizes the potential im-

One can expect consequent stabilization of HIF-α subunits and activation of HIF signalling upon UCP up-regulation; however, to our knowledge no experimental data link together UCPs and HIF pathways [52]. Establishment of such a link is very attractive, since both UCPs and HIFs are largely involved in cancer development, including colon cancer [23,53].

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SUPPLEMENTARY ONLINE DATA

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**Figure S1** Baf increases cellular respiration in a concentration-dependent manner

Experiment was conducted in a hypoxia chamber at 6% atmospheric O₂. Asterisk indicates significant difference.

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A. V. Zhdanov, R. I. Dmitriev and D. B. Papkovsky

Figure S2 Treatment with Baf induces 3.5-fold increase in Fluo-4 intensity, reflecting strong elevation of cytosolic Ca\(^{2+}\).

Live cell confocal images of the cells treated with 0.25 μM Baf or mock (DMSO) for 1.5 h, z-stack of two planes. Bar represents 20 μm.

Figure S3 Baf-dependent stabilization of HIF-α in normoxia is substantially lower than in anoxia or on treatment with CoCl\(_2\).

Effects of Baf and CMA are similar. Inset, ratio between HIF levels upon treatment with Baf (0.25 μM) and CoCl\(_2\) (200 μM) is shown.

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Uncoupling with bafilomycin activates HIF signalling in cancer cells

Table S1 Primers used for analysis of gene expression by RT-PCR
ATCB, β-actin; LDHA, lactate dehydrogenase.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>VEGFA</td>
<td>5'-GTGTGCGCAGACAGTGCTCCAG-3'</td>
<td>5'-AGGAAGCTCATCTCTCTATGTGCTG-3'</td>
</tr>
<tr>
<td>GLUT1</td>
<td>5'-CAGCACGCTGTATGCCACCA-3'</td>
<td>5'-AAGCCGGAAAGCATCTCATCGA-3'</td>
</tr>
<tr>
<td>LDHA</td>
<td>5'-GTGCCATCATATCTAATAGAAGGACTTG-3'</td>
<td>5'-TCAAGCCACAGTGTAAGATATCC-3'</td>
</tr>
<tr>
<td>ATCB</td>
<td>5'-GAGCGGGAATCTCGTGCGTGACA-3'</td>
<td>5'-GTACATGTGTGGCGCCAGAC-3'</td>
</tr>
<tr>
<td>CAIX</td>
<td>5'-GGCGAGGAGGACCTGCGCCA-3'</td>
<td>5'-GTGGCCATGTGCGCCAGGC-3'</td>
</tr>
<tr>
<td>PHD2</td>
<td>5'-CAAGCTCGTGGCGAGGCA-3'</td>
<td>5'-GCCGCTCCTGAACTTCCCG-3'</td>
</tr>
<tr>
<td>PHD3</td>
<td>5'-CTGGAGCAGCGTGCAAGCAGCT-3'</td>
<td>5'-TTGGGTGTTCCAGTGCGCG-3'</td>
</tr>
<tr>
<td>PDK1</td>
<td>5'-TGGCGGAAGGATGTGCCGTCAG-3'</td>
<td>5'-GCTGGTGACAGATCCACCCA-3'</td>
</tr>
</tbody>
</table>

Table S2 Gene expression levels in HCT116 cells, WT (at 20.9 % O₂) and SC02−/− (at 6 and 20.9 % O₂)
Results are normalized to mock control (WT cells treated with DMSO), LDHA, lactate dehydrogenase.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Atmospheric O₂ level</th>
<th>Treatment</th>
<th>GLUT1</th>
<th>LDHA</th>
<th>CAIX</th>
<th>PHD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC02−/−</td>
<td>20.9%</td>
<td>DMSO</td>
<td>1 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baf</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DMOG</td>
<td>1.6 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.6</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6%</td>
<td>DMSO</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baf</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DMOG</td>
<td>1.6 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>WT</td>
<td>20.9%</td>
<td>DMSO</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baf</td>
<td>1.3 ± 0.2</td>
<td>1 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DMOG</td>
<td>1.7 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
</tbody>
</table>

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