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Organ Preservation Solutions Attenuate Accumulation and Nuclear Translocation of Hypoxia-inducible Factor-1 α in the Hepatoma Cell Line HepG2

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Keywords:	HIF-1 α , hepatocytes, organ preservation solution, nitric oxide, reactive oxygen species
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	<p>the tricarboxylic acid cycle – succinate, fumarate, malate – did not alter HIF-1α accumulation, although succinate exhibited a beneficial effect on cell viability in cold storage. In conclusion, preservation solutions attenuate accumulation and nuclear translocation of the transcription factor HIF-1α, and this property is seemingly related to their chemical composition (L-arginine, α-ketoglutarate). Thus, it appears feasible to design preservation solution specifically to modify HIF-1α accumulation and nuclear translocation.</p>



For Peer Review

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3 **Organ Preservation Solutions Attenuate Accumulation and Nuclear Translocation of**
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5 **Hypoxia-inducible Factor-1 α in the Hepatoma Cell Line HepG2**
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31 **Running head:** Preservation solutions and HIF-1 α accumulation
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Abstract

Hypoxia-inducible factor-1 α (HIF-1 α) is a key transcription factor orchestrating hypoxic and inflammatory reactions. Here, we determined the impact of organ preservation solutions (Celsior, histidine-tryptophan-ketoglutarate solution = HTK, University of Wisconsin solution = UW), oxygen supply and temperature on HIF-1 α accumulation, recorded by Western blotting and immunocytochemistry, in the human hepatoma cell line HepG2. Generation of reactive oxygen species (ROS), NO, and cell viability were concomitantly assessed. At 4°C, HIF-1 α accumulation was not detectable. In normothermic (37°C) cell culture medium (Dulbecco's Modified Eagle's Medium = DMEM), HepG2 cells accumulated HIF-1 α even in normoxia (21% O₂) which was not observed in either of the preservation solutions. This correlated to high generation of NO, a normoxic stabilizer of HIF-1 α , and L-arginine content (substrate for NO synthesis) in DMEM, and low NO production and absence of L-arginine in preservation solutions. In normothermic hypoxia up to 24 h, intracellular HIF-1 α accumulated in all conditions, but less in preservation solutions compared to DMEM. The inhibitory effect on accumulation and nuclear translocation was most prominent for HTK, the only solution containing the activator of HIF-1 α degradation, α -ketoglutarate. Addition of other intermediates of the tricarboxylic acid cycle – succinate, fumarate, malate – did not alter HIF-1 α accumulation, although succinate exhibited a beneficial effect on cell viability in cold storage. In conclusion, preservation solutions attenuate accumulation and nuclear translocation of the transcription factor HIF-1 α , and this property is seemingly related to their chemical composition (L-arginine, α -ketoglutarate). Thus, it appears feasible to design preservation solution specifically to modify HIF-1 α accumulation and nuclear translocation.

KEY WORDS: HIF-1 α ; hepatocytes; organ preservation solution; Celsior; histidine-tryptophan-ketoglutarate solution; University of Wisconsin solution; nitric oxide; reactive oxygen species

INTRODUCTION

Organ transplantation is unavoidably accompanied by ischemia-reperfusion and periods of tissue hypoxia, causing cellular damage and initiating a broad spectrum of adaptive cellular responses. Hypoxia-inducible factor-1 α (HIF-1 α) is a key transcription factor orchestrating these reactions. HIF-1 α is constitutively produced under normoxia, but constantly degraded at high rate. Under hypoxic conditions, its degradation is prevented by a reduced action of HIF-prolyl-hydroxylases that require oxygen.¹⁻⁴ Hence, HIF-1 α accumulates under hypoxic conditions within minutes and governs transcription of genes encoding for proteins exerting beneficial effects under hypoxia, e.g. by improving anaerobic energy production.^{5,6} Seen under the aspect that HIF-1 α prepares cells to survive in a hypoxic environment, gene expression under the control of HIF-1 α is considered as a potential therapeutic target for improving graft outcome.⁷⁻⁹

There is, however, another facet of HIF-1 α . Besides its prominent role in hypoxia-induced reactions such as increased glycolysis and angiogenesis, it is also essential for myeloid cell-mediated inflammation.^{10,11} The conditional loss of HIF-1 α in a genetically engineered mouse strain completely inhibited the inflammatory response, i.e. leukocyte infiltration and edema formation, in three different *in vivo* models of inflammation.¹⁰ Moreover, HIF-1 α has been linked to cancer metastasis and matrix invasion.¹² Accordingly, seen under the pro-inflammatory and tumour promoting aspects, HIF-1 α exerts adverse effects and strategies to prevent its accumulation or to block its transcriptional coactivation have been suggested for therapeutic use.^{13,14}

Thus, accumulation of HIF-1 α may have opposite consequences on acute and long-term cellular survival, probably also depending on the type of tissue injury/diseases. In any case, however, its impact on conditions of ischemia-reperfusion and inflammation is significant, and consequently, it has moved in the focus of interest in transplantation research. Originally, HIF-1 α has been taken as a surrogate marker of local hypoxia, and elevated mRNA and

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3 protein levels have been detected in ischemic grafts and in cadaveric donor organs.¹⁵⁻¹⁷ The
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5 impact of surgical and pharmacological strategies on HIF-1 α accumulation in grafts, however,
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7 is just at the beginning of being investigated. Recently, it has been demonstrated that ischemic
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9 preconditioning of donor livers by 10-min inflow occlusion (Pringle maneuver) followed by
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11 10 min of reperfusion immediately prior to the initiation of cold ischemia increased nuclear
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13 HIF-1 α content compared to the control group.¹⁸
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17 Along this line, we set out to determine the impact of the use of various preservation solutions
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19 (Celsior, histidine-tryptophan-ketoglutarate solution = HTK, and University of Wisconsin
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21 solution = UW) and temperature on HIF-1 α accumulation in the human hepatoma cell line
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23 HepG2. In view of the pronounced modulatory effect of reactive oxygen species (ROS) and
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25 nitric oxide (NO) on HIF-1 α accumulation^{19,20}, their generation was also assessed in the
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27 present set of experiments. The HepG2 cell culture model was chosen because 1) it avoids
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29 uneven distribution of oxygen tension within the sample as it is inherently present in tissues
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31 and organs, thereby adding another variable to the interpretation of the results, and 2) HepG2
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33 cells represent a widely used and well characterized model to study hypoxia-driven cellular
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35 reactions including HIF-1 α stabilization in human cells.²⁰⁻²² It has previously been shown that
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37 certain metabolites of the tricarboxylic acid cycle (TCA) improve organ function when delivered
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39 at hypoxia (α -ketoglutarate, fumarate, malate)²³ and are also linked to HIF-1 α metabolism.
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41 HIF-prolyl-hydroxylases require α -ketoglutarate and generate succinate^{1,2,4}, and the
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43 equilibrium of α -ketoglutarate, succinate, and fumarate controls stabilization and nuclear
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45 translocation of HIF-1 α .^{6,24} Hence, the impact of addition of these TCA intermediates to
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47 preservation solutions on HIF-1 α accumulation was also analyzed.
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MATERIALS AND METHODS

Cell culture

Cell line: HepG2 cells are a cell line derived from a human hepatocellular carcinoma and were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM High Glucose with L-Glutamine; PAA Laboratories, Cölbe, Germany) containing 10% foetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin in an atmosphere of 5% CO₂ at 37°C. All supplements were obtained from PAA Laboratories GmbH (Cölbe, Germany). In the following this complete medium is designated as "DMEM". One to 3 days before the start of the tests, cells were seeded in DMEM into 25 cm² culture flasks (Nunc - Thermo Fisher Scientific, Wiesbaden, Germany) for Western blotting experiments, into 8 well culture slides (Falcon, Heidelberg, Germany) for immunocytochemistry or analysis of ROS-, NO-production, respectively, and into 96 well plates (Cellstar; Greiner bio-one, Frickenhausen, Germany) to perform an MTT-assay. Cell density was 0.4-0.8 x 10⁵ cells/cm²

Organ preservation solutions: At the beginning of the experiments, HepG2 cells were washed twice with the corresponding organ preservation solution and then covered with Celsior (Genzyme, Neu-Isenburg, Germany), HTK (Dr. Franz Köhler Chemie, Alsbach-Hähnlein, Germany), or UW (Bristol-Myers Squibb, Munich, Germany). Controls were kept in DMEM. In a separate series of experiments the organ preservation solutions were supplemented with malate, fumarate or succinate (all from Sigma-Aldrich, Taufkirchen, Germany) at final concentrations of 15 mM. This concentration was chosen because of its reported beneficial effect on ischemia-reperfusion of the rat heart.²³

Normothermic or hypothermic incubation at normoxia or hypoxia: Cultures were incubated for the indicated times at warm normoxia (21% O₂, 5% CO₂, 37°C), warm hypoxia (1% O₂, 5% CO₂, 37°C), cold normoxia (room air, 4°C), or cold hypoxia (1% O₂, 5% CO₂, 4°C).

Western Blotting

Preparation of cell extracts: Cells seeded into culture flasks were incubated for 6 h, 12 h or 24 h at normothermic or hypothermic normoxia or hypoxia. Then the cultures were washed twice with cold phosphate-buffered saline (PBS) and scraped with a rubber policeman (Techno Plastic Products AG, Trasadingen, Switzerland) from the bottom of the flasks. Detached hepatocytes of one flask were diluted in 5 ml PBS and transferred into a 15 ml tube. Cells were sedimented by centrifugation (2500 rpm, 4°C, 5 min), resuspended in 500 µl PBS and transferred into an Eppendorf tube. Again, cells were sedimented by centrifugation and the weight of the pellet was determined. The 5fold volume of extraction buffer (7 M urea, 10% glycerol, 10 mM Tris-HCl pH 6.8, 1% sodium dodecyl sulphate (SDS), 5 mM dithiothreitol, 0.5 mM PMSF, 1x concentrated Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) was added and cells were disintegrated by a ball mill (Mixer Mill MM300; Retsch, Haan, Germany).

Western blotting: Protein solutions were resolved by 10% SDS-polyacrylamide electrophoresis and transferred onto a nitrocellulose membrane (Millipore, Schwalbach, Germany). After incubation with blocking buffer (Tris-buffered saline = TBS, 0.01% Tween 20, 10% milk powder) for 60 min at room temperature, the membranes were covered overnight at room temperature with mouse monoclonal anti-human HIF-1 α antibody (clone 54, BD Transduction, Heidelberg, Germany; diluted 1:333 in TBS, 0.01% Tween 20, 5% milk powder). Then the membranes were washed with TBS, 0.01% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Pierce, Rockford, IL, USA; 1:10,000 in TBS, 0.01% Tween 20, 2.5% milk powder). After 1 h, unbound antibodies were removed by three washes with TBS, 0.01% Tween 20. Bound antibodies were visualized by the Super Signal West Pico Chemiluminescent Substrate Kit (Pierce). For the densitometric evaluation on each PVDF membrane the band with the strongest signal was encircled with a

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3 well fitting rectangle and the mean grey value within this area was calculated on a scale of
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5 gray values ranging from 0 to 255 using open-to-public software “ImageJ 1.37V”.
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7 Subsequently, the other bands were outlined by rectangles of identical size and the grey
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9 values were analysed, too. For standardization, a pooled extract of cells subjected to
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11 normothermic hypoxia in DMEM for various time points in a separate set of experiments was
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13 prepared, and this sample was run as a standard in each blot. The densitometric gray value of
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15 this sample was set as 100%.
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19 The effect of the addition of malate, fumarate or succinate to the organ preservation solutions
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21 on HIF-1 α stabilization was analyzed in HepG2 cells incubated for 6 or 24 h at hypoxia,
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23 37°C.
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28 29 **Immunocytochemistry**

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31 HepG2 cells seeded into 8 well culture slides and covered with DMEM, Celsior, HTK or UW
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33 were exposed to normoxia or hypoxia at 37°C. After 6 h, the cultures were briefly washed
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35 with PBS and fixed for 15 min with 4% buffered formaldehyde in PBS. The fixative was
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37 removed by washing with PBS and cells were permeabilized with PBS containing 0.1%
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39 Triton X-100 (10 min). After another washing step with PBS, cultures were covered for 1 h
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41 with blocking medium (PBS, 10% normal horse serum, 0.5% Tween 20, 0.5% bovine serum
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43 albumin) followed by overnight incubation with a mouse monoclonal anti-human HIF-1 α
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45 antibody (clone 54, BD Transduction; 1:250). The cells were washed with PBS and covered
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47 with Cy3-conjugated donkey anti-mouse IgG (Dianova, Hamburg, Germany; 1:2,000). After
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49 three washes with PBS, the cells were fixed in 4% buffered formaldehyde for 15 min at room
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51 temperature. The fixative was rinsed off with PBS and 1 μ g/ml 4',6-diamidino-2-phenylindole
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53 (DAPI; Sigma-Aldrich) in PBS was added for labelling of the nuclei. After 15 min the cells
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55 were washed three times with PBS, coverslipped in Mowiol medium and evaluated with a
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60 Zeiss Axioplan 2 epifluorescence microscope.

Detection of ROS and NO

Generation of ROS was assayed with 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma Aldrich). After intracellular cleavage of the diacetate by endogenous esterases and oxidation by ROS, the compound turns into a highly fluorescent product. 4,5-Diaminofluorescein diacetate (DAF-2DA; Alexis Biochemicals, Grünberg, Germany) was used to analyse NO production. After entering the cell, DAF-2DA is hydrolyzed to DAF-2, which is converted in the presence of NO into the highly fluorescent triazolofluorescein (DAF-2T). For detection of ROS and NO generation, HepG2 cells were seeded into 8 well culture slides, covered with DMEM and organ preservation solutions, respectively, and incubated for 6, 12 or 24 h at warm/cold normoxia/hypoxia. Thirty min before the end of the incubation time DCF-DA (final concentration: 20 μ M) or DAF-2DA (final concentration: 10 μ M) was added to the solutions. As negative controls cultures in DMEM without fluorescence indicators were used. At the end of the incubation, cells were washed twice with normoxic and hypoxic pre-gassed solutions, respectively. Then cells were fixed with 4% formaldehyde which was previously gassed with N₂ for fixation of the cells incubated at hypoxia. Subsequently, cells were washed with PBS, coverslipped in Mowiol medium (Fluka, Buchs, Switzerland) and evaluated with a Zeiss Axioplan 2 epifluorescence microscope equipped with an AxioCam camera (Zeiss). To prevent ROS formation during the evaluation, specimens were stored at 4°C. The fluorescence signal intensity of individual cells was determined by the open-to-public software "ImageJ" and expressed as relative gray values at a scale of 0-255. Per condition fifty cells were evaluated and for further statistical analysis the median was taken. The effect of the addition of malate, fumarate or succinate to Celsior, HTK or UW on ROS or NO generation was analyzed for 12 h at normothermic or hypothermic normoxia and hypoxia, respectively.

MTT Assay

For analysis of viability, HepG2 cells were seeded into 96 well plates. For each condition, 5 wells were prepared. Before exposure to normoxia or hypoxia cells were washed with the appropriate organ preservation solution and then covered with this solution (150 μ l/well). As controls cells cultured in DMEM (standard cell culture medium for HepG2) or in DMEM containing 100 μ M H₂O₂ (inducer of cell death) were used. After incubation at normothermic/hypothermic normoxia/hypoxia for 6 h, all cultures were maintained for an additional hour at normoxia, 37°C, to bring all cells to a comparable metabolic status. Then the supernatants were discharged and replaced by 100 μ l DMEM containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT; Sigma-Aldrich). After 120 min at normoxia, 37°C, supernatants were removed and cells were dissolved in 75 μ l 0.1 N HCl (Merck, Darmstadt, Germany) diluted in isopropanol (Merck). Optical densities (OD) were measured at 570 nm and 690 nm, and the ratio OD 570 nm/OD 690 nm as well as the difference between OD 570 nm and OD 690 nm were calculated. From the 5 values obtained per condition the median was calculated and used for further statistical analysis.

Statistical analysis

Statistical analysis was performed by using SPSS Base 15 (SPSS Software, Munich, Germany). Percentiles 0, 25, 50, 75 and 100 are presented in box plots. Differences among experimental groups were first analyzed with the non-parametric global Kruskal-Wallis test. If this test revealed $p \leq 0.05$, subsequent analysis between two groups was performed with the non-parametric Mann-Whitney test. Throughout, $p \leq 0.05$ was considered significant and $p \leq 0.01$ highly significant.

RESULTS

Accumulation and nuclear translocation of the transcription factor HIF-1 α

Basal stabilization of HIF-1 α even under normoxia at 37°C was noted when HepG2 cells grew in cell culture medium, DMEM, but not or only minimally when they were maintained in any of the preservation solutions (Fig. 1). At 6 h of normothermic hypoxic incubation, HIF-1 α was significantly stabilized (Fig. 1) and translocated into the nucleus (Fig. 2). Immunocytochemistry revealed strong hypoxia-induced accumulation of HIF-1 α in the nucleus and nearly no cytoplasmic HIF-1 α in cells kept in DMEM (Fig. 2). This sharp contrast between nuclear and cytoplasmic distribution was less pronounced in cells maintained in preservation solutions (Fig. 2). Specifically, nuclear HIF-1 α was lowest in cells kept in HTK solution (Fig. 2). In general, a high degree of HIF-1 α stabilization persisted up to 24 h. In HTK solution, however, a significant reduction of HIF-1 α content was noted at 24 h of normothermic hypoxic incubation, being no longer significantly different to the corresponding normoxic control (Fig. 3). In all of these conditions, the absolute amount of HIF-1 α protein was consistently found to be higher in DMEM compared to any of the preservation solutions (Figs. 1-3). Among them, there were no significant differences, except between Celsior and HTK at 24 h of hypoxic incubation at 37°C. Neither of the TCA cycle intermediates (malate, fumarate, succinate) had any impact on HIF-1 α stabilization after 6 (not shown) and 24 h of hypoxic incubation at 37°C (Fig. 4).

At 4°C, stabilization of HIF-1 α protein was undetectable at each incubation condition tested (Figs. 1, 3).

ROS generation

Oxidative conversion of DCF-DA into DCF, resulting in intracellular fluorescence above baseline was observed in all incubation conditions, both at 37°C and at hypothermia (4°C) (Fig. 5). In general, highest DCF fluorescence intensities at 37°C were observed after culturing in Celsior solution at all time points investigated (6, 12 and 24 h), whereas no

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3 significant differences were noted between DMEM, HTK and UW solution. In hypoxia at
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5 4°C, DCF fluorescence increased in DMEM over time, reaching significant difference to
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7 hypoxia at 37°C after 24 h only. At this time point only, there was a significant difference to
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9 any of the preservation solutions (lower signal in UW solution, Fig. 5). In neither of the
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11 media, hypoxic incubation significantly altered ROS production compared to normoxia at the
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13 same temperature. Data obtained for 24 h of incubation are displayed in figure 5, those
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15 resulting from other time points are not shown. The impact of TCA cycle intermediates on
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17 ROS generation was investigated at 12 h of incubation. Except a slight inhibiting effect of
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19 fumarate on hypoxic ROS generation in Celsior solution at 37°C, no significant differences
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21 between various conditions were observed (Fig. 6, additional data for UW solution not
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23 shown).
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32 **NO generation**

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34 Conversion of DAF-2DA into the fluorescent triazole derivative DAF-2T resulting in
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36 intracellular fluorescence above baseline was observed in all incubation conditions.
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38 Throughout, highest fluorescence intensities were observed in cells maintained in DMEM. In
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40 normoxia at 37°C, this difference was statistically significant for all preservation solutions
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42 and all time points studied (6, 12 and 24 h). In hypoxia at 37°C, this difference was
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44 statistically significant for UW solution and Celsior, and in hypoxia at 4°C it was significant
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46 for UW solution only, except for 6 h of incubation where all solutions differed from DMEM.
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48 In organ preservation solutions, there was a general trend towards increased DAF-2T
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50 fluorescence in hypothermia which reached significance for HTK solution at 12 and 24 h of
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52 normoxic and hypoxic incubation. Data obtained for 24 h of incubation are displayed in figure
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54 7, those resulting from other time points are not shown. Neither of the TCA cycle
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56 intermediates had a significant impact on NO generation at 12 h of incubation (data not
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58 shown).
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Cell viability

Cell viability was assessed by the colorimetric MTT test and recorded as the OD differences at wavelengths 570 nm and 690 nm. This assay clearly demonstrated superior viability of HepG2 cells in DMEM cultured for 6 h at 37°C. Low viability in DMEM was observed only in room air (normoxia) at 4°C where alkalization of DMEM occurs because its buffer composition requires 5% CO₂ (Fig. 8). After culturing at 37°C, lowest values were observed for UW (Fig. 8). Notably, hypoxic incubation did not significantly reduce viability of HepG2 cells.

Among TCA cycle intermediates, succinate significantly elevated MTT conversion in 5 out of 6 hypothermic conditions (exception: UW solution, hypoxia), and fumarate was beneficial in 2 hypothermic conditions (Celsior at normoxia, UW at hypoxia) (Fig. 8). At normothermia, neither of the intermediates significantly altered cell viability (data not shown).

DISCUSSION

The present data clearly document an impact of organ preservation solutions on intracellular accumulation and nuclear translocation of HIF-1 α , a transcription factor centrally involved in regulation of inflammation and adaptation to hypoxia. Under normal conditions, HIF-1 α is constantly produced by cells, but rapidly subjected to proteasomal degradation so that HIF-1 α protein is practically undetectable by routine methods. Regulation of the intracellular amount of HIF-1 α mainly takes place at the level of HIF-1 α hydroxylation which targets the protein to degradation.^{6,25} Hypoxia prevents hydroxylation and, thereby, causes HIF-1 α accumulation, but there are other pathways operating at normoxic conditions that also end up in HIF-1 α stabilization, sometimes referred to as “pseudohypoxia”.²⁴ Both hypoxic and “pseudohypoxic” HIF-1 α accumulation occurs in cultured human hepatoma HepG2 cells, as the present data demonstrate.

Notably, all organ preservation studies investigated here markedly suppressed both pathways, hypoxic and normoxic (“pseudohypoxic”), of HIF-1 α accumulation. Specifically, in case of normoxia, i.e. 21% O₂ in the gaseous phase of the incubator, this suppression was so strong that HIF-1 α protein was entirely undetectable. Normoxic stabilization of HIF-1 α can be achieved by NO and ROS, also depends on cell density, and these pathways interfere with each other.^{20,26,27,28} Hence, altered ROS and/or NO production by cells kept in organ preservation solutions might provide a clue for the suppressive effect upon HIF-1 α accumulation. Indeed, normoxic NO production at 37°C was significantly higher in conventional cell culture medium (DMEM) than in any of the preservation solutions. This difference most probably reflects the availability of L-arginine, the substrate for NO synthases, in DMEM whereas all preservations solutions do not contain this amino acid. Low NO synthesis was particularly marked for HTK and UW, in which median values of NO-indicator fluorescence were about 25% of that seen in DMEM. In Celsior, reduced NO production was by far not that prominent. However, a concomitant elevated ROS-indicator

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3 fluorescence was observed in this particular solution only, and it has been previously shown
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5 that ROS attenuate NO-mediated HIF-1 α stabilization in HepG2 cells.²⁰ Collectively, the
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7 specific patterns of NO and ROS production would predict a presence of normoxic HIF-1 α
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9 stabilization in cells kept in DMEM but an absence in organ preservation solutions, and this
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11 matches the actual observations.
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15 In hypoxia, reversed effects of NO and ROS upon HIF-1 α stabilization are reported in that
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17 both of them partially counteract hypoxia-induced accumulation of HIF-1 α protein¹⁹, but
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19 opposing data for ROS also have been presented.²⁹ Hence, it is difficult to interpret the
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21 presently observed differences of hypoxic ROS and NO production between cell culture
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23 medium and preservations solutions with respect to their potential to contribute to diminished
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25 HIF-1 α accumulation in cells kept in preservation solutions. Both increases and decreases of
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27 NO and ROS production have been reported under various experimental hypoxic conditions,
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29 and it is still a matter of debate whether ROS are centrally linked to hypoxia-induced gene
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31 transcription.^{6,30,31} Noteworthy, in neither of the solutions normothermic hypoxia *per se*
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33 caused alterations in NO- and ROS-indicator fluorescence in our experiments, strongly
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35 favouring a direct effect of reduced oxygen tension on activity of HIF-prolyl-hydroxylases¹⁻⁴
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37 rather than involving NO or ROS as intermediates.
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44 Besides molecular oxygen, HIF-prolyl-hydroxylases require α -ketoglutarate as a substrate,
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46 and they are inhibited by their product, succinate, as well as by fumarate.^{32,33} Accordingly,
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48 direct proof has been provided that elevated α -ketoglutarate promotes HIF-1 α degradation
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50 whereas elevated succinate and fumarate levels promote HIF-1 α accumulation.^{33,37} In our
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52 experiments, addition of succinate, fumarate or malate to preservation solutions did not
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54 interfere with HIF-1 α content, nor did it influence NO and ROS production. The reasons for
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56 this are currently unclear and may include ineffective uptake of these TCA intermediates by
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58 HepG2 cells, despite the observation that their application to heart perfusates clearly had
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60 marked intracellular effects.²³ Interestingly, it has been shown recently by Mailloux et al.

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3 (2009), that nuclear accumulation of HIF-1 α in hepatocytes exposed to aluminium was
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5 ablated by addition of 5 mM α -ketoglutarate to the culture medium. They further
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7 demonstrated that aluminium-mediated nuclear accumulation of HIF-1 α is connected to TCA
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9 cycle metabolism.³⁷ This fits well to our observation of missing nuclear localisation of HIF-1 α
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11 in HepG2 cells cultured in HTK, the only organ preservation solutions containing α -
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13 ketoglutarate.
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17 However, much in line with the requirement of α -ketoglutarate for HIF-1 α degradation
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19 initiated by HIF-prolyl-hydroxylation, we observed lowest hypoxic HIF-1 α accumulation in
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21 the nucleus and a drop in the time course of the experiment in cells kept in HTK, the only
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23 preservation solution containing α -ketoglutarate (1 mmol/l). These data strongly indicate that
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25 the chemical composition of preservation solutions can shape cellular HIF-1 α metabolism.
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29 The strongest single determinant of HIF-1 α accumulation was temperature. While generation
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31 of NO and ROS, as determined by fluorescent indicators, continued at 4°C both at normoxia
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33 and hypoxia and was even elevated in some conditions³⁸, HIF-1 α protein was generally
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35 undetectable after cold storage, even after 24 h in hypoxia. These data indicate that in clinical
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37 practice even prolonged cold liver storage is unlikely to lead to HIF-1 α stabilization *per se*.
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41 While the present data provide direct evidence that three commonly used preservation
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43 solutions attenuate cellular HIF-1 α accumulation, and that this effect can be modified by their
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45 chemical composition, neither the present cell culture study nor previous reports can answer
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47 the question whether HIF-1 α is finally beneficial or injurious in clinical organ transplantation.
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51 Here, we assessed cell viability by the MTT test after 6 h of experimental incubation and a
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53 subsequent incubation for 1 h at 37°C and 21% O₂. When preincubated at 37°C, cell viability
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55 was best in DMEM, the medium in which cells achieved highest HIF-1 α levels, being
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57 consistent with the view that HIF-1 α triggers cellular adaptation to hypoxia thereby promoting
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59 cell survival.¹⁸ However, a direct causal relationship has not been investigated and more
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general toxic effects of histidine and phosphates contained in Celsior, HTK and UW have

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3 very recently been claimed to cause cell injury to cultured rat hepatocytes.³⁹ With respect to
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5 the addition of TCA cycle intermediates, we achieved a better readout in the MTT test after
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7 supplementation of cold preservation solutions with succinate, but this was apparently
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9 unrelated to HIF-1 α since its accumulation did not occur at all at 4°C, and even at 37°C was
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11 unaffected by succinate. Particularly in view of the pro-inflammatory effects of HIF-1 α (10),
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13 this transcription factor may play opposite roles in clinical transplantation in the acute phase
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15 of organ ischemia and transport and in organ rejection, issues that can not be addressed by the
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17 present cell culture model but deserve further investigation.
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22 In conclusion, organ preservation solutions attenuate normoxic and hypoxic intracellular
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24 accumulation and nuclear translocation of the transcription factor HIF-1 α in a human
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26 hepatoma cell line. This general characteristic as well as differences among the preservation
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28 solutions are seemingly related to their chemical composition (arginine, α -ketoglutarate). On
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30 this basis it appears feasible to design preservation solutions specifically to modify HIF-1 α
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32 accumulation and nuclear translocation. Further *in vivo* experimental and clinical studies on
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34 long-term beneficial or deleterious consequences of HIF-1 α triggered reactions in organ
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36 transplantation, however, are necessary to decide whether this newly opened strategy shall be
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38 used to further suppress or to elevate HIF-1 α accumulation.
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45 46 **Acknowledgment**

47
48 We gratefully thank Ms A. Goldenberg for skilled technical assistance and Ms K. Michael for
49
50 expert figure layout.
51
52

53 54 55 **REFERENCES**

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FIGURE LEGENDS

Figure 1. Detection of HIF-1 α protein by Western blotting after incubation of HepG2 cells for 6 h at the conditions indicated in the figure. At warm normoxia (21% O₂), HIF-1 α accumulates only in cells grown in DMEM culture medium. At warm hypoxia (1% O₂), a 120 kDa HIF-1 α -immunoreactive band is also seen in cells kept in preservation solutions Celsior, HTK and UW, but less intense than in DMEM. No HIF-1 α protein is detectable after cold incubation of cells. *Standard* designates a sample that was run in every blot and which was set as 100% in densitometric evaluation. Molecular weight markers are indicated at the left.

Figure 2. Detection of HIF-1 α protein by immunocytochemistry in HepG2 cells incubated at 37°C for 6 h under normoxic or hypoxic conditions. Nuclei are labelled by the DNA-binding blue fluorophore, DAPI. In normoxia, modest cytoplasmic and nuclear HIF-1 α immunofluorescence is visible in cells grown in DMEM. After hypoxic incubation, a rise in immunofluorescence is seen in all solutions. Nuclear translocation of HIF-1 α is particularly evident in DMEM, modestly present in Celsior and UW, but barely visible in HTK. The specificity of the secondary antibody detection system is demonstrated by absence of labelling when primary antibody is omitted (lower panel). The specificity of the primary antibody was validated by Western blotting (cf. Figure 1).

Figure 3. Quantification of HIF-1 α accumulation in HepG2 cells as determined by densitometric evaluation of Western blots. Throughout, the same pooled standard extract was set as 100%. Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x standard deviation. Differences in normoxic HIF-1 α stabilization at 37°C between DMEM on the one hand and all preservation solutions (left 3 bars in each panel) are evident and statistically highly significant throughout ($p \leq 0.01$). Statistically significant differences between

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3 corresponding normoxic and hypoxic conditions for each solution are indicated in the figure,
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5 with * $p \leq 0.05$, ** $p \leq 0.01$, and n.s. = not significant. HTK is the only solution in which HIF-1 α
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7 accumulation is elevated at 6 and 12 h, but then turns down after 24 h of normothermic
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9 hypoxia. At 4°C, no accumulation of HIF-1 α is seen throughout.
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14
15 Figure 4. Quantification of HIF-1 α accumulation in HepG2 cells as determined by
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17 densitometric evaluation of Western blots. Cells were kept in hypoxia for 24 h at 37°C.
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19 Addition of fumarate, malate and succinate had no impact on HIF-1 α accumulation.
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21 Throughout, the same pooled standard extract was set as 100%. Data are presented in box
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23 plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small
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25 boxes represent data beyond 3x standard deviation. N.s. = not significant, Kruskal-Wallis test.
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32 Figure 5. Detection of ROS generation in HepG2 cells by the fluorescent indicator DCF-DA
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34 after incubation for 24 h. At 37°C, normoxic and hypoxic ROS generation in Celsior, but not
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36 in other preservation solutions, were higher than in DMEM. N = normoxia (21% O₂), H =
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38 hypoxia (1% O₂). Background fluorescence that is not due to DCF-DA conversion by ROS
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40 was determined in cells that were incubated without indicator fluorophore (DCF-DA \emptyset). Data
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42 are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75,
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44 and 100. Small circles and boxes represent data beyond 3x standard deviation. Throughout,
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46 number of experiments was 6-7. * $p \leq 0.05$, ** $p \leq 0.01$
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53 Figure 6. Detection of ROS generation in HepG2 cells by the fluorescent indicator DCF-DA
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55 after incubation in Celsior for 12 h. Addition of malate (M), fumarate (F), and succinate (S)
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57 had no impact on ROS formation, except a reducing effect of fumarate in hypoxia at 37°C. \emptyset
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59 = Celsior without further supplementation. Data are presented in box plots depicting
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percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes

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3 represent data beyond 3x standard deviation. Number of experiments was 5-6, except for
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5 normoxia, 4°C (n=3). *p≤0.05
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10 Figure 7. Detection of NO generation in HepG2 cells by the fluorescent indicator DAF-2DA
11 after incubation for 24 h. At 37°C, normoxic and hypoxic NO generation in DMEM is higher
12 than in preservation solutions ([#]p≤0.05 and ^{##}p≤0.01, compared to the corresponding DMEM
13 group). N = normoxia (21% O₂), H = hypoxia (1% O₂). Differences between normothermia
14 and hypothermia within a group are indicated by brackets (*p≤0.05, **p≤0.01). Background
15 fluorescence that is not due to DAF-2DA conversion by NO was determined in cells that were
16 incubated without indicator fluorophore (DAF-2DA Ø). Data are presented in box plots
17 depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles
18 and boxes represent data beyond 3x standard deviation. Numbers of experiments were 3-4 for
19 hypoxia at 4°C in DMEM and HTK, and 5-8 in all other conditions.
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36 Figure 8. Assessment of cell viability by MTT test after 6 h of incubation under the conditions
37 indicated in the figure. The *upper panel* depicts raw data of optical densities (OD),
38 demonstrating higher viability in DMEM at 37°C. Significant differences to DMEM are
39 indicated by brackets (*p≤0.05, **p≤0.01). At normoxia, 4°C, cells were incubated in room
40 air, causing pH shift in DMEM with resulting low viability. To illustrate the impact of
41 addition of malate (M), fumarate (F), and succinate (S) in the *lower panel*, within each group
42 the OD value measured in the respective solution without supplementation (Ø) was set as
43 100%. Hy = Samples which were treated with H₂O₂ to control for the ability of the assay to
44 detect cell damage. Data are presented in box plots depicting percentiles 0, 25, 50 (median,
45 indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x
46 standard deviation. Number of experiments was 6 in each group.
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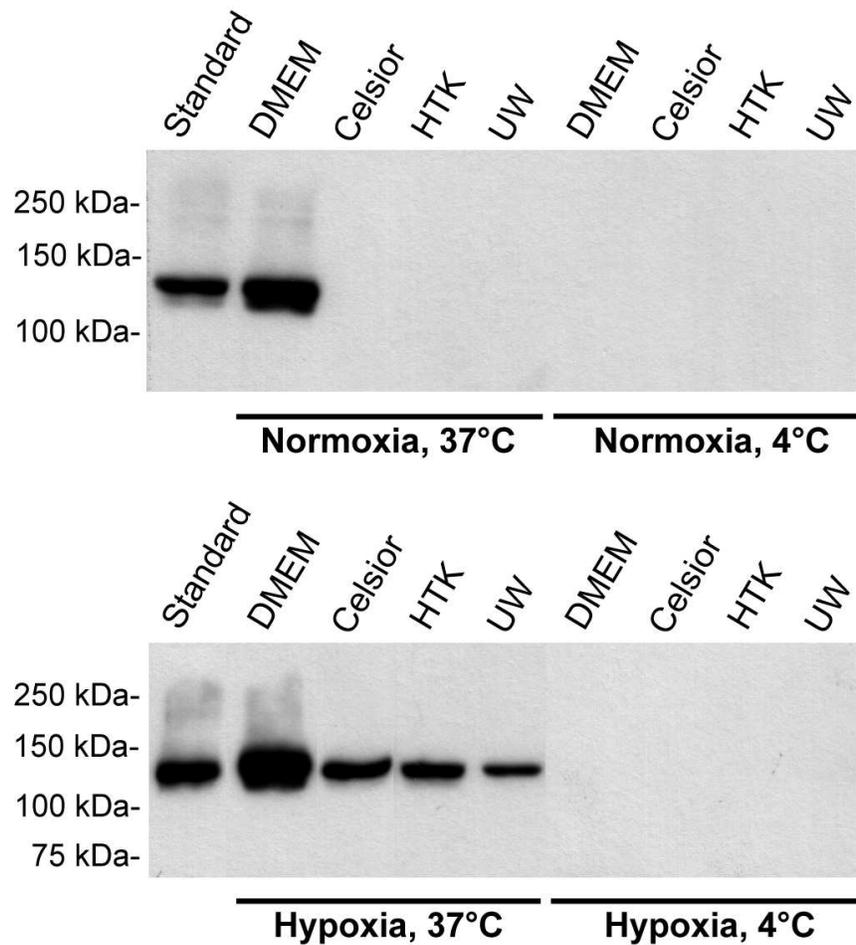


Fig. 1

Figure 1. Detection of HIF-1 α protein by Western blotting after incubation of HepG2 cells for 6 h at the conditions indicated in the figure. At warm normoxia (21% O₂), HIF-1 α accumulates only in cells grown in DMEM culture medium. At warm hypoxia (1% O₂), a 120 kDa HIF-1 α -immunoreactive band is also seen in cells kept in preservation solutions Celsior, HTK and UW, but less intense than in DMEM. No HIF-1 α protein is detectable after cold incubation of cells. Standard designates a sample that was run in every blot and which was set as 100% in densitometric evaluation. Molecular weight markers are indicated at the left.

89x121mm (300 x 300 DPI)

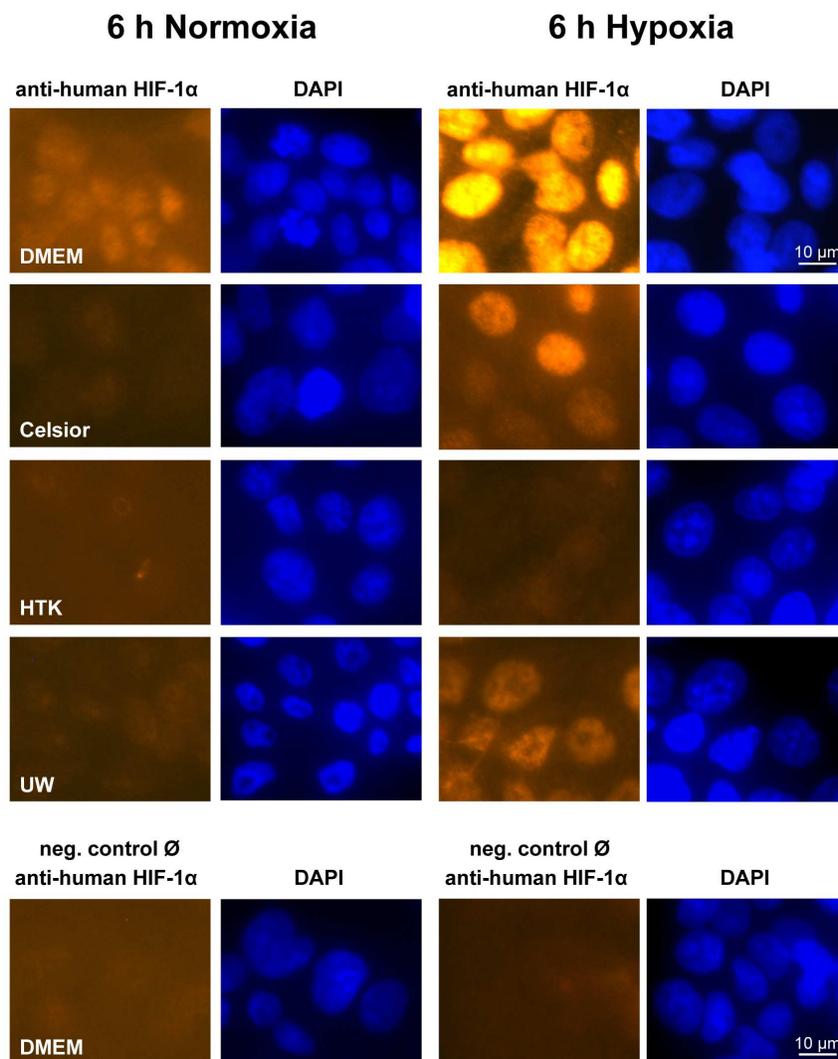


Fig. 2

Figure 2. Detection of HIF-1 α protein by immunocytochemistry in HepG2 cells incubated at 37°C for 6 h under normoxic or hypoxic conditions. Nuclei are labelled by the DNA-binding blue fluorophore, DAPI. In normoxia, modest cytoplasmic and nuclear HIF-1 α immunofluorescence is visible in cells grown in DMEM. After hypoxic incubation, a rise in immunofluorescence is seen in all solutions. Nuclear translocation of HIF-1 α is particularly evident in DMEM, modestly present in Celsior and UW, but barely visible in HTK. The specificity of the secondary antibody detection system is demonstrated by absence of labelling when primary antibody is omitted (lower panel). The specificity of the primary antibody was validated by Western blotting (cf. Figure 1).

146x202mm (300 x 300 DPI)

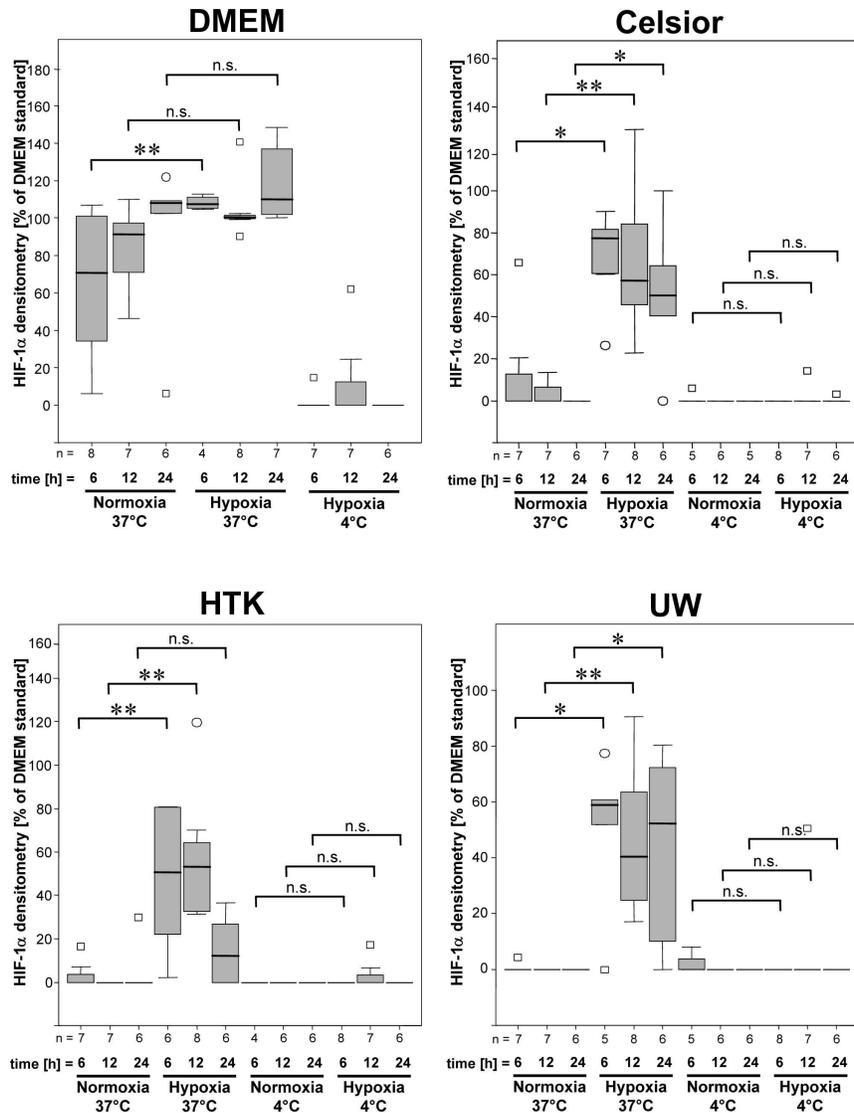


Fig. 3

Figure 3. Quantification of HIF-1 α accumulation in HepG2 cells as determined by densitometric evaluation of Western blots. Throughout, the same pooled standard extract was set as 100%. Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x standard deviation. Differences in normoxic HIF-1 α stabilization at 37°C between DMEM on the one hand and all preservation solutions (left 3 bars in each panel) are evident and statistically highly significant throughout ($p \leq 0.01$). Statistically significant differences between corresponding normoxic and hypoxic conditions for each solution are indicated in the figure, with $*p \leq 0.05$, $**p \leq 0.01$, and n.s. = not significant. HTK is the only solution in which HIF-1 α accumulation is elevated at 6 and 12 h, but then turns down after 24 h of normothermic hypoxia. At 4°C, no accumulation of HIF-1 α is seen throughout.

172x237mm (300 x 300 DPI)

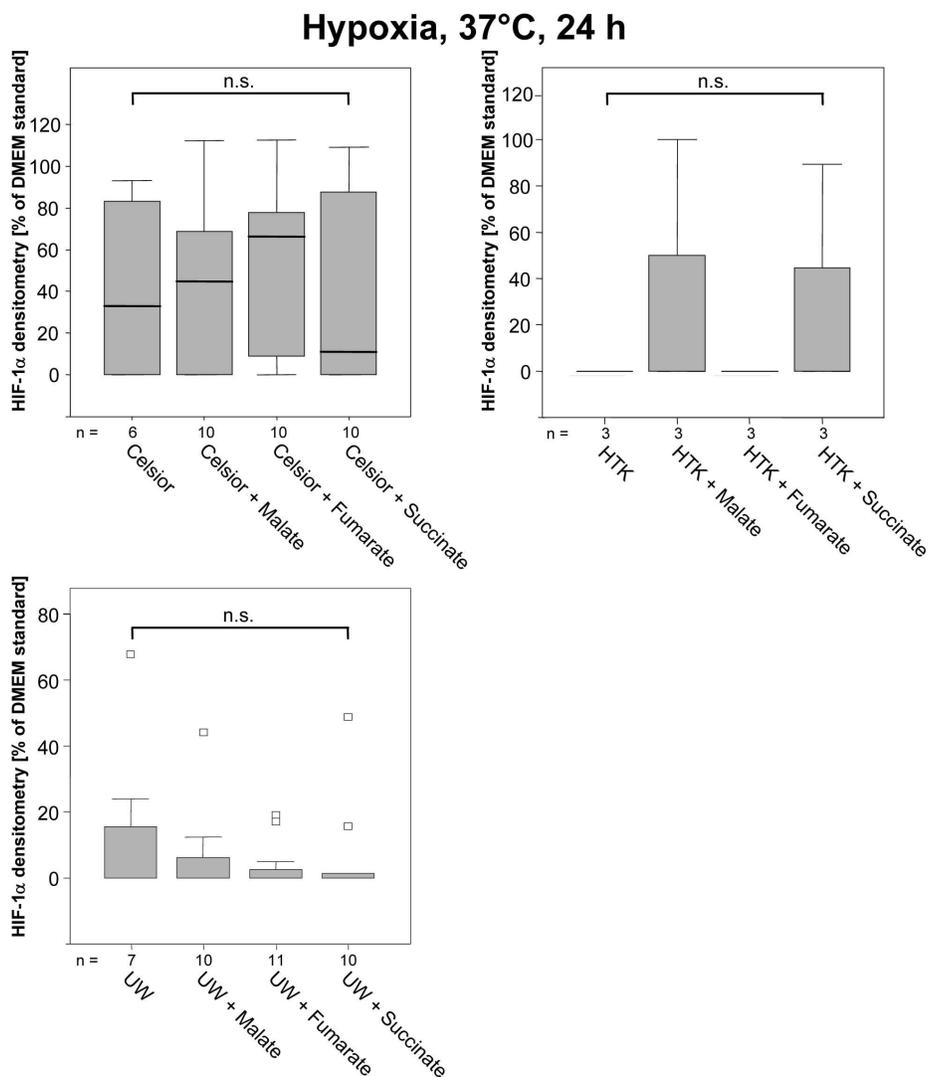


Fig. 4

Figure 4. Quantification of HIF-1 α accumulation in HepG2 cells as determined by densitometric evaluation of Western blots. Cells were kept in hypoxia for 24 h at 37°C. Addition of fumarate, malate and succinate had no impact on HIF-1 α accumulation. Throughout, the same pooled standard extract was set as 100%. Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small boxes represent data beyond 3x standard deviation. N.s. = not significant, Kruskal-Wallis test.
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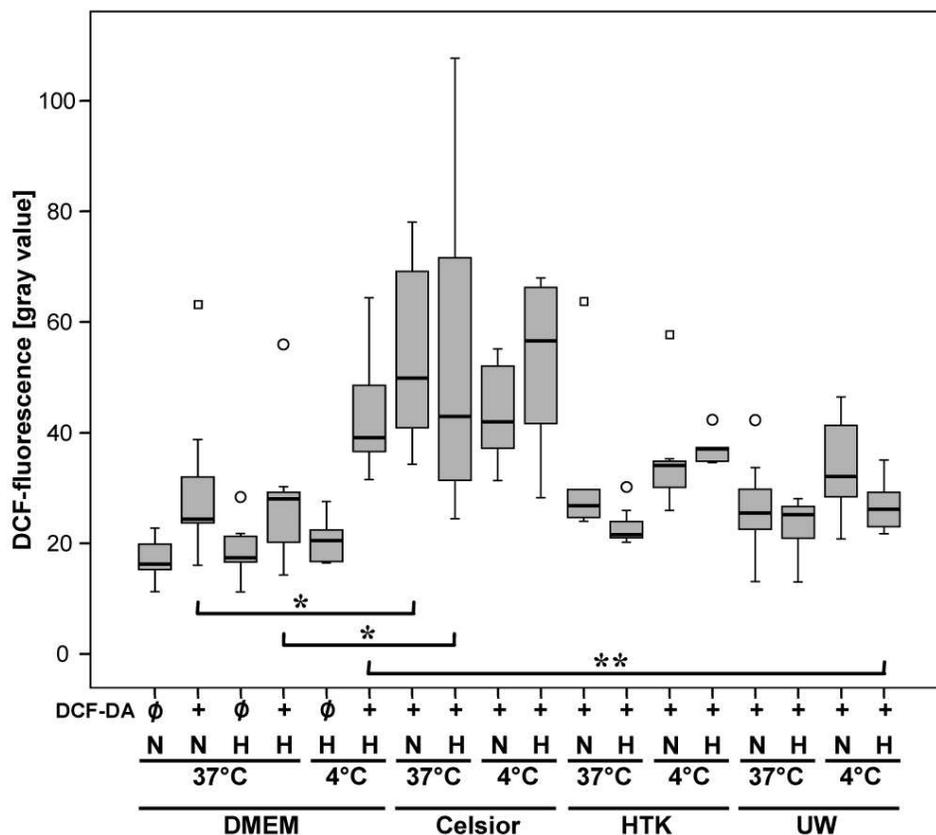


Fig. 5

Figure 5. Detection of ROS generation in HepG2 cells by the fluorescent indicator DCF-DA after incubation for 24 h. At 37°C, normoxic and hypoxic ROS generation in Celsior, but not in other preservation solutions, were higher than in DMEM. N = normoxia (21% O₂), H = hypoxia (1% O₂). Background fluorescence that is not due to DCF-DA conversion by ROS was determined in cells that were incubated without indicator fluorophore (DCF-DA ∅). Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x standard deviation. Throughout, number of experiments was 6-7.

*p≤0.05, **p≤0.01

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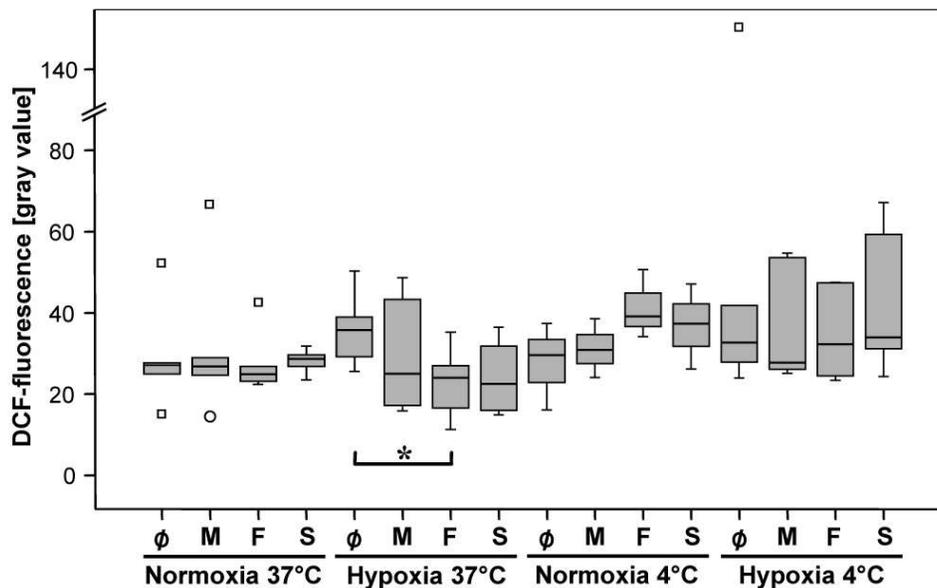


Fig. 6

Figure 6. Detection of ROS generation in HepG2 cells by the fluorescent indicator DCF-DA after incubation in Celsior for 12 h. Addition of malate (M), fumarate (F), and succinate (S) had no impact on ROS formation, except a reducing effect of fumarate in hypoxia at 37°C. Ø = Celsior without further supplementation. Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x standard deviation. Number of experiments was 5-6, except for normoxia, 4°C (n=3). *p<0.05
89x76mm (300 x 300 DPI)

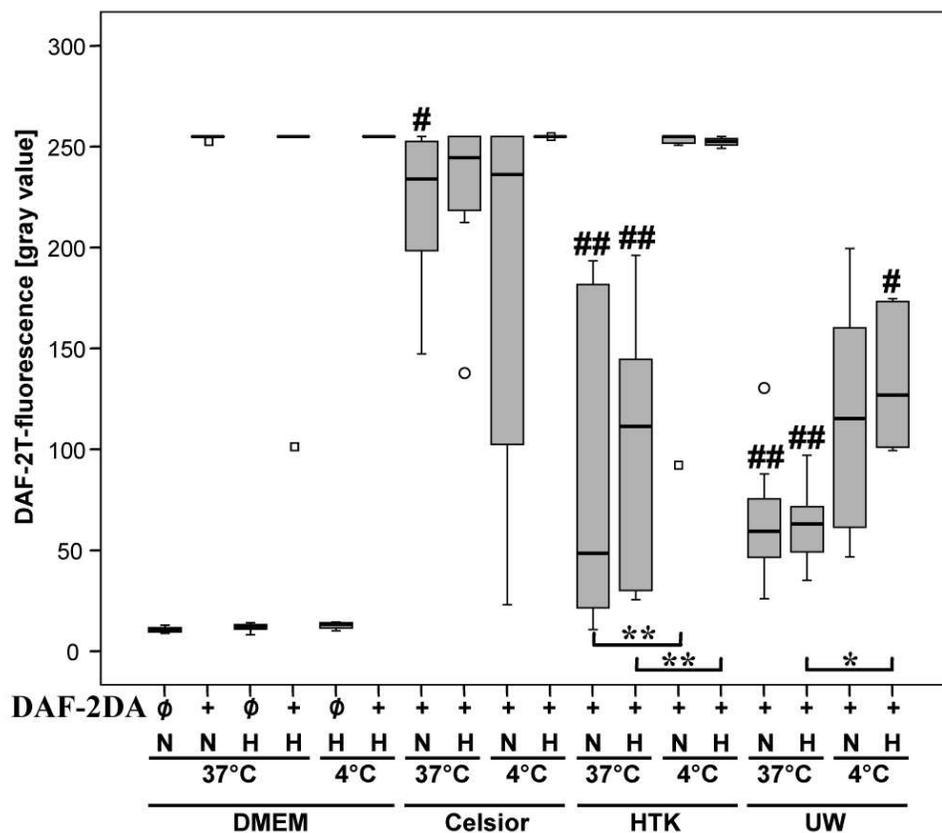


Fig. 7

Figure 7. Detection of NO generation in HepG2 cells by the fluorescent indicator DAF-2DA after incubation for 24 h. At 37°C, normoxic and hypoxic NO generation in DMEM is higher than in preservation solutions ($\#p \leq 0.05$ and $\#\#p \leq 0.01$, compared to the corresponding DMEM group). N = normoxia (21% O₂), H = hypoxia (1% O₂). Differences between normothermia and hypothermia within a group are indicated by brackets ($*p \leq 0.05$, $**p \leq 0.01$). Background fluorescence that is not due to DAF-2DA conversion by NO was determined in cells that were incubated without indicator fluorophore (DAF-2DA Ø). Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x standard deviation. Numbers of experiments were 3-4 for hypoxia at 4°C in DMEM and HTK, and 5-8 in all other conditions.

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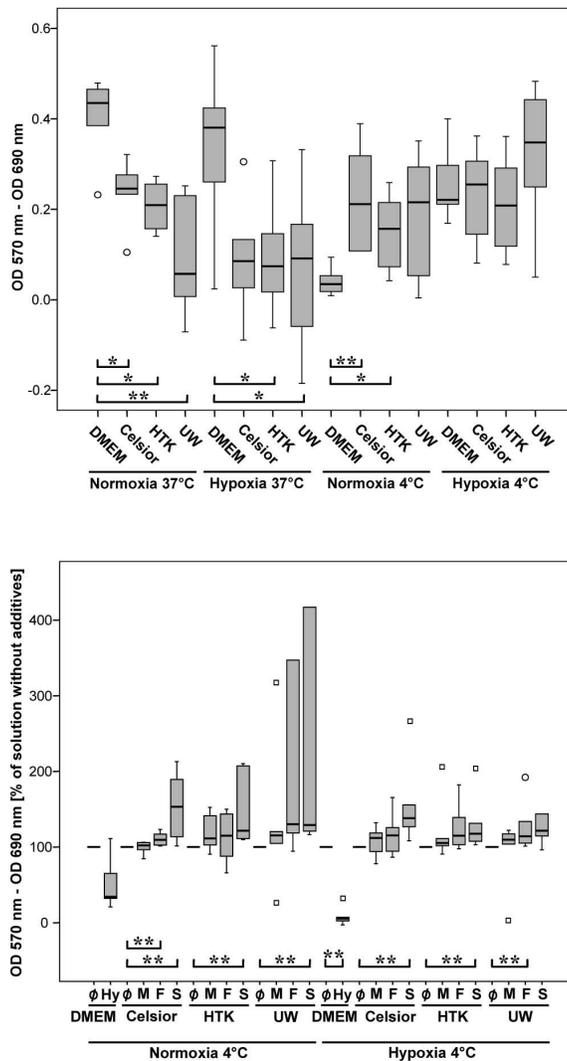


Fig. 8

Figure 8. Assessment of cell viability by MTT test after 6 h of incubation under the conditions indicated in the figure. The upper panel depicts raw data of optical densities (OD), demonstrating higher viability in DMEM at 37°C. Significant differences to DMEM are indicated by brackets (* $p \leq 0.05$, ** $p \leq 0.01$). At normoxia, 4°C, cells were incubated in room air, causing pH shift in DMEM with resulting low viability. To illustrate the impact of addition of malate (M), fumarate (F), and succinate (S) in the lower panel, within each group the OD value measured in the respective solution without supplementation (\emptyset) was set as 100%. Hy = Samples which were treated with H₂O₂ to control for the ability of the assay to detect cell damage. Data are presented in box plots depicting percentiles 0, 25, 50 (median, indicated by black bar), 75, and 100. Small circles and boxes represent data beyond 3x standard deviation. Number of experiments was 6 in each group.

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