SUPPLEMENTARY MATERIAL

To Be Or Not to Be: the "Smoker's Paradox" – An in-Vitro Study

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Supplementary Material and Methods

Quantification of cell death

Using Annexin-V/Propidium-Iodide double staining based flow cytometry analyses the type of cell death induced was determined. Therefore HL-1 cells were cultured in 6-well plates (250.000 HL-1 cells per well) and treated as indicated. When indicated, 20µM Z-VAD-FMK (Enzo Life Sciences, Switzerland) was added in order to inhibit caspase-dependent apoptosis; inhibition of necrosis was induced with addition of 30µM Necrostatin-1 (Sigma Aldrich, Germany).

Presence of reactive oxygen species (ROS)

HL-1 cells were seeded in 6-well plates and treated as indicated. After 23h of hypoxia, 0h, 1h, 23h, 47h, 71h and 95h of reperfusion, cells were incubated with 5µM 1, 2, 3-Dihydrorhodamine (123-DHR) according to the manufacturer's instructions (Sigma-Aldrich, Germany). After another hour either in the hypoxic chamber or in the incubator, HL-1 cells were detached, washed in PBS without Calcium and Magnesium (PBS -/-) (Sigma-Aldrich, Germany) and analyzed using flow cytometry techniques (FACS Canto II, BD) by recording the median values of fluorescence intensity. When indicated 1mM of N-Acetyl-L-Cysteine (NAC) was added. Incubation of cells with NAC was performed during hypoxia and the indicated reoxygenation times. When indicated, 0.5mM of H_2O_2 was added.

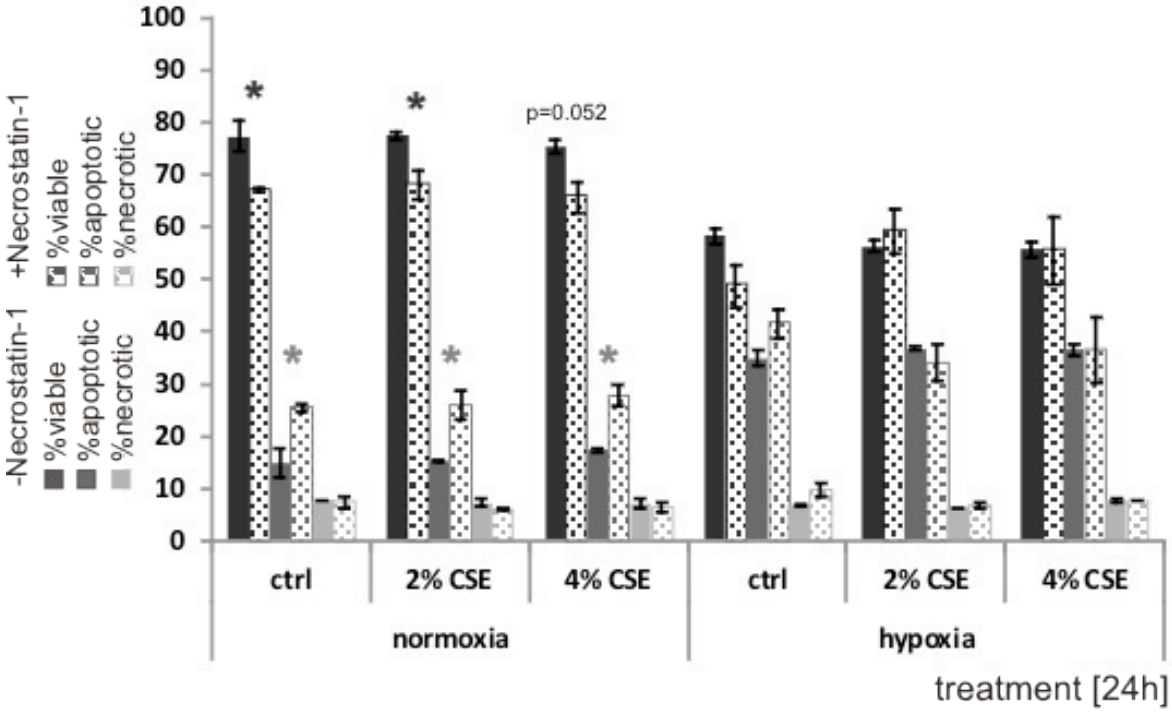
Determination of protein content

HL-1 cells were seeded in culture dishes (24h CSE: 2.000.000 cells per dish; 48h CSE: 1.500.000 cells per dish) and treated as indicated. Cells were removed using a cell scraper, centrifuged and each probe resuspended in 100µl lysis buffer (1ml RIPA buffer[1], 1µl Pepstatin, 1µl Leupeptin, 1µl Aprotinin, 10µl AEBSF). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Thermo Fischer Austria).

Supplementary Figure I

Addition of Necrostatin-1 does not rescue CSE induced HL-1 cell death

To determine whether or not the occurring necrosis involves the receptor-interacting-protein-kinase-1 (RIPK-1) the experiment was repeated with addition of Necrostatin-1, an inhibitor of RIPK-1. No biologically relevant effect was observed (Suppl. Fig. I).

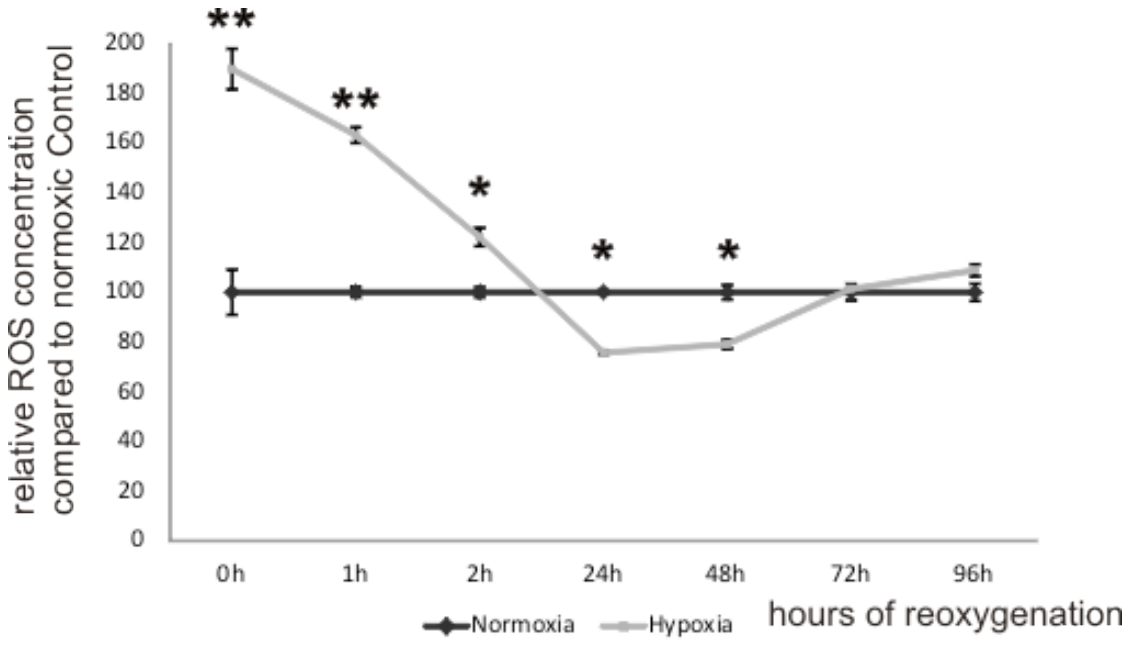


Suppl. Fig. I Effect of CSE on HL-1 cell death with addition of Necrostatin-1. HL-1 cells were incubated with different concentrations of CSE for 24h with addition of Necrostatin-1 (inhibitor of RIPK-1 induced necrosis), subjected to the hypoxia/reperfusion model, stained with Annexin-V/PI and analyzed using FACS techniques. Data expressed as mean ± SD. * $p<0.05$

Supplementary Figure II

Production of ROS in Reoxygenation

To investigate, if hypoxic conditions indeed reduce ROS presence, 123-DHR staining of HL-1 cells subjected to hypoxia and different times of reoxygenation (0h, 1h, 24h, 48h, 72h, 96h) was performed. In the first two hours of reoxygenation ROS are present, then diminish in the following hours until oxidative stress at 24h and 48h of reoxygenation is indeed lower than under normoxic conditions. After longer periods of reoxygenation, oxidative stress again rises to normoxic levels (Suppl. Fig. II).

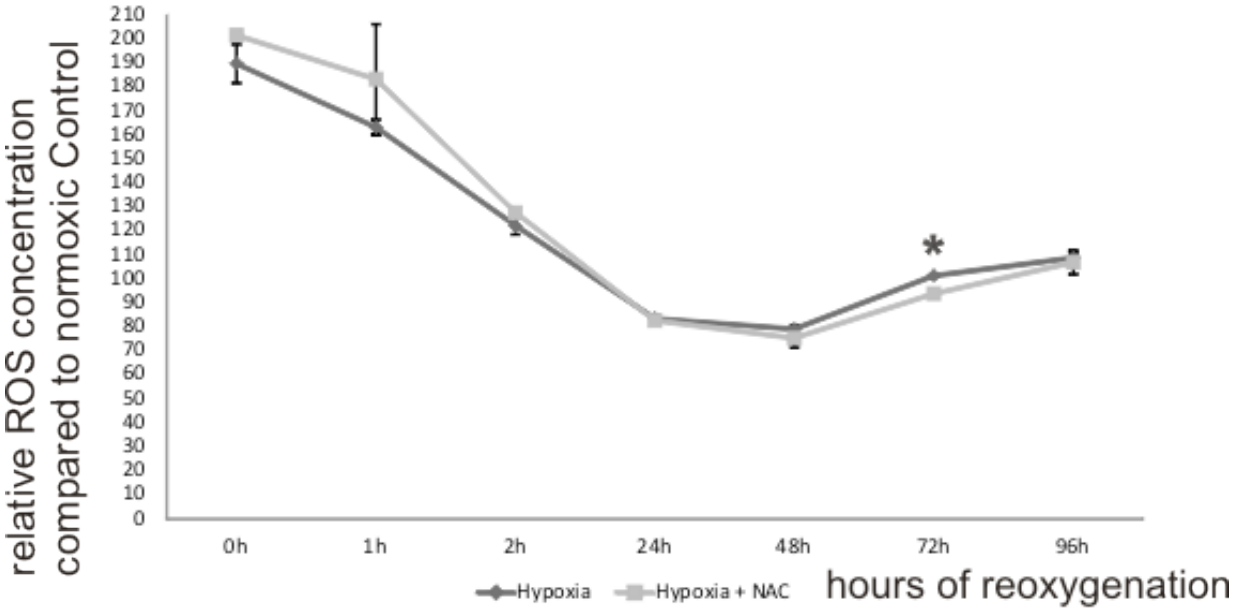


Suppl. Fig. II ROS production at different times of Reoxygenation. HL-1 cells were subjected to 24h of hypoxia and indicated times of reoxygenation, stained with 123-DHR and analyzed using flow cytometry techniques. Data are collected as median values of fluorescence intensity± SD as percent in relation to normoxic control * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Supplementary Figure III

Addition of NAC does not influence presence of ROS

To evaluate whether or not NAC is capable of alleviating hypoxia induced HL-1 cell death, 123-DHR staining with addition of NAC was performed. NAC did not relevantly decrease hypoxia/reperfusion induced oxidative stress (Suppl. Fig. III).

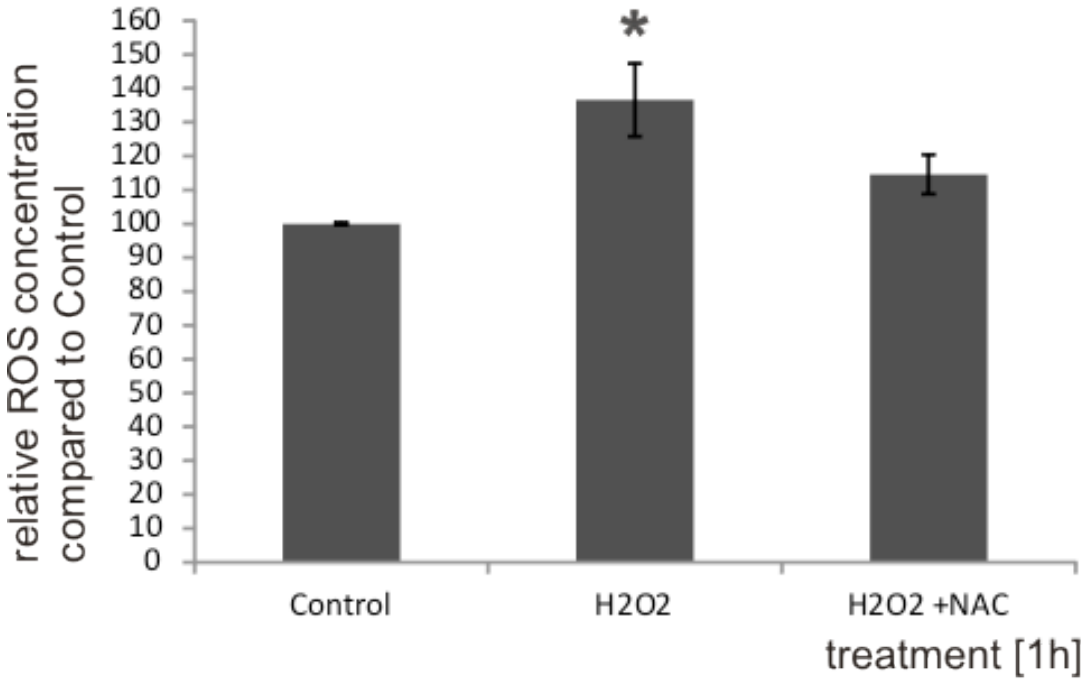


Suppl. Fig. III Effect of NAC on ROS generation. HL-1 cells were subjected to 24h of hypoxia with addition of NAC, stained with 123-DHR and analyzed using flow cytometry techniques. Data expressed as median ± SD in relation to normoxic control * $p<0.05$

Supplementary Figure IV

Addition of NAC effectively reduces Oxidative Stress induced by H_2O_2

A positive control with addition of H_2O_2 (incubation time 1h), which is known to produce oxidative stress, was performed. NAC was able to reduce oxidative stress induced by H_2O_2 (but not completely) (Suppl. Fig. IV).

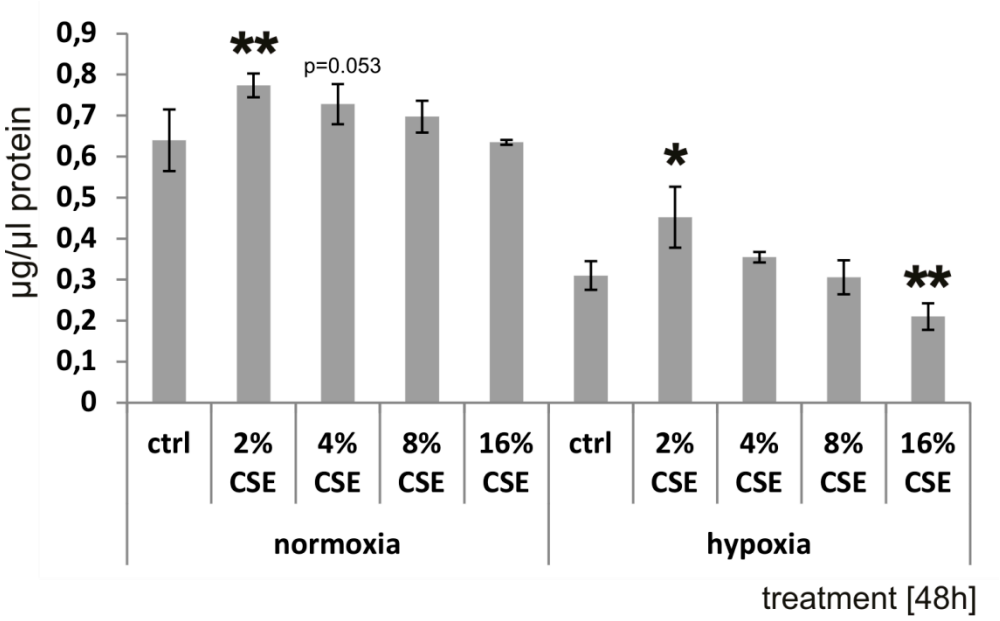


Suppl. Fig. IV Effect of H_2O_2 on ROS generation. HL-1 cells were incubated with H_2O_2 with addition of NAC, stained with 123-DHR and analyzed using flow cytometry techniques. Data expressed as median ± SD in relation to control * $p<0.05$

Supplementary Figure V

CSE incubation leads to increased amount of harvested protein

When collecting protein samples for western blot analyses, an increase in the amount of protein with addition of CSE was observed (Suppl. Fig. V).



Suppl. Fig. V Influence of CSE incubation on protein concentration. For Western Blot analyses, HL-1 cells were seeded in culture dish, incubated with different CSE concentrations for indicated times and subjected to the hypoxia/reperfusion model. Protein was extracted and quantified. Data expressed as mean ± SD * $p<0.05$; ** $p<0.01$

References

1 Habener A, Chowdhury A, Echtermeyer F, Lichtinghagen R, Theilmeier G, Herzog C: MitoNEET protects HL-1 cardiomyocytes from oxidative stress mediated apoptosis in an in vitro model of hypoxia and reoxygenation. PLoS One 2016;11:1–18.