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Original Paper

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

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Key Words

Smoker's Paradox • Myocardial infarction • Smoking • Necrosis-apoptosis switch • Cardiovascular diseases

Abstract

Background/Aims: Clinical studies have reported a better outcome of smokers after myocardial infarction compared to non-smokers. The data are controversial, as some clinical studies did not observe this effect. The cell biological processes involved, which might account for a 'Smoker's Paradox', have not been investigated yet. Therefore, the aim was to elucidate the effect of cigarette smoke on the viability of cardiomyocytes in the context of hypoxia and reperfusion. ***Methods:*** HL-1 cells were incubated with different concentrations of cigarette smoke extract (CSE) and subjected to hypoxia/reperfusion to further evaluate influence of CSE on viability of HL-1 cells using flow cytometry analyses, Western Blot and immunofluorescence staining. ***Results:*** Incubation with CSE led to a concentration-dependent reduction in HL-1 viability. Adding hypoxia as a stressor enhanced cell death. Caspase-independent apoptosis was the observed type of cell death partly induced by P53 and apoptosis-inducing-factor. Yet a significant increase in LDH release in cardiomyocytes incubated with 4%, 8% and 16% CSE suggests necrosis with rapid DNA depletion. Interestingly, after hypoxia a decreased LDH release under lower CSE concentrations was observed. Moreover, a concentration-dependent increase in proliferation and a trend for increased ATP availability under hypoxic conditions was shown. ***Conclusions:*** The trend for less LDH release in hypoxia after low-level CSE incubation might represent a switch from necrosis to apoptosis, which in combination with the increase in metabolic activity and ATP availability might account for the 'Smoker's Paradox'. These findings could partly explain inconsistent results of previous clinical studies as the data showed strong evidence for the crucial relevance of the amount of cigarettes smoked. We are in need of future studies distinguishing between different types of smokers to finally verify or falsify the 'Smoker's Paradox'.

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Introduction

Cardiovascular diseases (CVDs) are still the number one cause of death in the western world [1]. In Europe the prevalence of death caused by CVDs is currently 40% in men and 49% in women [2]. Due to these high numbers in most western countries [3], intensive research is focused on a better understanding of the ongoing processes. Among CVDs, ischemic heart diseases also known as coronary artery diseases (CADs) are the most prevalent ones [4] and can be defined as insufficient oxygen delivery to the cardiac muscle due to coronary artery spasm, embolism or vasculitis but most commonly due to atherosclerotic plaques compromising the vessel lumen [5]. If blood flow and therefore oxygen supply in a large coronary artery is cut off, hypoxia at first results in reversible alterations of the myocardium, which turn into a state of irreversible damage after 20-40 minutes resulting in acute myocardial infarction (MI). This process involves necrosis of cardiomyocytes due to the generation of reactive oxygen species (ROS) causing oxidative stress to the surrounding tissue, which directly damages cell membranes and leads to cell death [6]. The goal is to restore blood flow to end hypoxia, which is once again associated with damage to the myocardium. The so-called reperfusion injury is characterized by enzyme release and death of cardiomyocytes. Necrosis is the major occurring type of cell death but evidence suggests apoptosis to predominate in the peripheral infarct lesion [7]. Various risk factors for MI have been established over the years. Next to a raised ApoB/ApoA1 ratio, current tobacco smoking is considered to be the strongest risk factor [8]. Paradoxically, it has been observed that smokers have lower mortality hence improved short-term outcome after acute MI compared to non-smokers known as the "Smoker's Paradox" [9, 10]. It has to be taken into consideration though that smokers presenting with acute MI are of younger age, have lower co-morbidities, often receive more aggressive treatment and have a lower overall risk profile than non-smokers presenting with acute MI. Some studies however claim a residual survival benefit for smokers even after multivariable adjustment [11]. Possible explanations are systematic errors due to publication bias, residual confounding due to lack of information about relevant risk factors or measurement errors, or different pathogenesis – which would represent a true effect of cigarette smoking [9]. Various theories exist about how to explain the "Smoker's Paradox". Smokers compared to non-smokers have lower oxygen saturation [12] and Jensen et al. suggest that smokers experience tissue hypoxia after tobacco smoking [13]. This could indicate periodical ischemia of cardiomyocytes, which might induce ischemic preconditioning effects. Moreover, it has been shown that smokers with STEMI compared to non-smokers with STEMI (ST-elevation myocardial infarction) have a better myocardial perfusion after fibrinolysis. This might be associated with the fact that tobacco smoking leads to increased levels of circulating fibrinogen and tissue factor, which suggests that a thrombus in smokers with STEMI would contain more fibrinogen and therefore be more amenable to fibrinolysis, resulting in a higher survival rate [9]. However, in today's therapeutic approach this mechanism has become less relevant since percutaneous coronary intervention (PCI) can be used with increasing frequency. More recent studies such as the SYNTAX trial, which compared PCI with coronary artery bypass grafting (CABG), found no evidence for the "Smoker's Paradox"[14]. De Luca et al. compared smokers to non-smokers after PCI concerning their infarct size and did not find any differences either [15]. Whereas Cohen et al. found that smokers had lower rates of subsequent target lesion revascularization, meaning repeated revascularization via PCI or CABG caused by clinical restenosis [16]. However, none of these studies searched for underlying cell biological mechanisms that might account for the "Smoker's Paradox". Therefore, our aim was to investigate the effect of CSE on the viability of cardiomyocytes under normoxic and hypoxic conditions.

Materials and Methods

Cell culture and treatment

HL-1 cardiomyocytes were provided by William C. Claycomb and cultured as described in Claycomb et al. [17] using Claycomb medium (Sigma-Aldrich, Germany). Cells were incubated with 2%, 4%, 8% and 16% CSE for 24 or 48h as indicated. Hypoxia-reperfusion conditions were simulated using culture medium without FCS and elimination of oxygen (<1%, determined by measurement of oxygen concentration) by addition of nitrogen in a hypoxic chamber (Modular Incubator Chamber, MIC-101, Billups-Rothenberg, USA). After 24h cells were once again supplied with FCS and oxygen and evaluated after another 24h or 72h of reoxygenation (Fig. 1A-C).

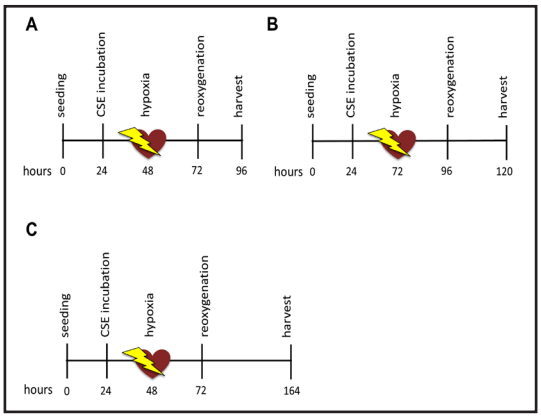

Fig. 1. Schematic representation of the hypoxia/reperfusion model A The basic design: 24 hours after HL-1 cells were seeded, cells were incubated with freshly made CSE for 24 hours, subjected to 24h of hypoxia and consecutively reoxygenated for another 24 hours to be harvested B Modification of design: 48 hours of CSE incubation C Modification of design: 72 hours of reoxygenation.

Preparation of Cigarette Smoke Extract (CSE)

CSE was freshly made using a "smoking machine", which was kindly provided by Priv. Doz. Dr. David Bernhard (Johannes Kepler University Linz). The setup and proper use of this machine has already been described elsewhere [18, 19]. The cigarette brand used was Marlboro Red. Concentrations used were 2, 4, 8 and 16% CSE.

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.

Quantification of intracellular DNA content

HL-1 cells were seeded in 6-well plates, incubated with increasing concentrations of CSE for indicated times and subjected to the hypoxia/reperfusion model. After enzymatic detachment of cells, HL-1 cells were permeabilized using Saponin (1mg/ml), DNA was stained using Propidium-Iodide (50µg/ml) and quantified using flow cytometry techniques (FACS Canto II, BD).

Evaluation of metabolic activity

An XTT-Assay (Biomol, Hamburg, Germany) was used to quantify the metabolic activity of viable cells in the exposed cultures to evaluate the proliferative activity of the cells. HL-1 cells were seeded in 96-well plates and treated as indicated.

Western blotting and immunodetection

HL-1 cells were seeded in cell 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 was resuspended in 100µl lysis buffer (1ml RIPA buffer [20], 1µl Pepstatin, 1µl Leupeptin, 1µl Aprotinin, 10µl AEBSF). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Thermo Fischer Austria). Equal amounts of protein were loaded onto polyacrylamide gels, separated and subsequently blotted using an iBlot 2 dry blotting system (Thermo Fischer, Austria). Antibodies used were anti-P53 (P53 antibody, biorbyt, UK; Cat.no.: orb11210) and anti-Cyclin D1 (Anti-Cyclin D1 antibody [SP4], abcam, UK; Cat.no.: ab16663); secondary antibody used was Anti-rabbit IgG, HRP-linked antibody (Cell Signaling, USA; Cat.no.: #7074). For the detection of protein oxidation, protein lysates were first derivatized, according to the manufacturer's instructions (OxyBlotTM, Millipore, Germany), loaded onto a 12% polyacrylamide

gel, separated and blotted, incubated with a primary and secondary antibody. CCCP treated cells were used as control. Further the membrane was treated with chemiluminescent reagents and exposed to an autoradiography film. Equal protein loading was controlled by Ponceau staining. Quantification of Western Blots was performed by measuring the band area using ImageJ [21].

In-vitro DNA laddering

For analysis of cytosolic nuclease activity of HL-1 cells incubated with CSE and subjection to hypoxia/reperfusion, nuclear DNA was extracted from HL-1 cells and incubated with cytosolic extracts of treated HL-1 as described before [22].

Presence of reactive oxygen species (ROS)

HL-1 cells were seeded in 6-well plates and treated as indicated. After 23h reperfusion, cells were incubated with 5µM 1, 2, 3-Dihydrorhodamine (123-DHR) according to the manufacturer's instructions (Sigma-Aldrich, Germany). After another hour 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).

Mitochondrial Depolarization Measurements

HL-1 cells were seeded in 6-well plates and treated as indicated. Changes in mitochondrial membrane potential were detected as previously described in Messner et al. [23].

Lysosomal labeling

For staining the acidic compartments in HL-1 cells, LysoTracker Green (Invitrogen, Molecular Probes, Cat.no.: L7526) and LysoSensor Green (Invitrogen, Molecular Probes, Cat.no.: L7535) were used. After the indicated treatment cells were stained with LysoTracker dye (75nM) for 30min or LysoSensor dye (75nM) for 5min at 37°C and analyzed using flow cytometry techniques.

Quantification of LDH release

Release of cellular LDH (Lactatdehydrogenase) was quantified using the LDH-Cytotoxicity Assay Kit II (Biovision, USA). Cells were seeded in 96-well plates (6000/well) and treated as indicated. Total LDH concentration and LDH released was measured and set in relation. The assay was performed as described in the user's manual instructions with minor adaptions (50µl reaction mix + 50µl PBS was added to each well).

Quantification of ATP availability

Availability of ATP (Adenosine Triphosphate) was quantified using the ATP Determination Kit (Invitrogen, Molecular Probes, UK) as described in the user's manual instructions and correlated with the protein concentration determined using a BCA Protein Assay Kit (Pierce, UK).

Scanning electron microscopic analysis

Cells were seeded in 6-well plates on glass coverslips and treated as indicated. After reperfusion, cells were fixed by first adding 5% glutaraldehyde in PBS -/- (Sigma-Aldrich, Germany) to the medium o/n and then replacing the medium with 2.5% glutaraldehyde in PBS -/- (Sigma-Aldrich, Germany). Following fixation, cells were washed with PBS, dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, three times 100%) 20 minutes each, desiccated chemically using Hexamethyldisilazane (HMDS, Sigma-Aldrich, Germany; Cat.No.: 379212) for 45 minutes and again for 10 minutes with fresh HMDS, HMDS was pipetted off and cover slips were allowed to dry over night. Next, cells were mounted (Silver DAG 1415, Christine Gröpl, Austria, Cat.No.: G3692) and sputtered with gold-palladium (Polaron CA 508; Fisons Instruments, Mainz-Kastel, Germany) and then examined with a JEOLJSM-5400 scanning electron microscope (Eching, Germany).

Immunofluorescence staining

Cells were seeded on Lab-Tek chamber slides (130.000 HL-1 cells per chamber) and treated as indicated. Medium was removed and cells were washed with PBS followed by staining with Mitotracker Red CMX-ROS (Invitrogen, UK) when indicated. Next, cells were fixed using 4% paraformaldehyde in PBS for 1.5 minutes,

once again washed with PBS, permeabilized with 0.1% Tween in PBS for 30 minutes and then incubated with 1% bovine serum albumin (BSA) in PBS to block nonspecific binding sites. Cells were incubated with the first antibody o/n; antibodies used were anti-apoptosis-inducing-factor (Anti-AIF antibody [E20], abcam, UK; Cat.No.: ab32516; 1:100 in 1% BSA in PBS) and anti-α-myosin heavy chain (anti-heavy chain cardiac Myosin, Abcam, UK; Cat.No.:ab15; 1:100 in 1% BSA in PBS). After three washing steps, cells were incubated with the second antibody (anti-AIF: Alexa Fluor 488, goat anti-rabbit, Molecular Probes, UK, Cat. No.: A11001; 1:1000 in PBS; anti-α-MHC: Alexa Fluor 488, goat anti-mouse, Molecular Probes, UK, Cat. No.: A11001; 1:800 in PBS) for 60 minutes in the dark at room temperature. The monolayer was washed again three times and cell nuclei were stained using either TOPRO-3 (Invitrogen, UK) (1:300 in PBS) for 20min or PI (1:200 in PBS) for 1.5min in the dark at room temperature. After three final washing steps, cells were mounted in ProLong Antifade and analyzed using a LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software (Zeiss, Germany). As a negative control, untreated HL-1 cells were washed, fixed and permeabilized analogous to the cells stained with the antibody. After blocking with 1% BSA in PBS for 30 minutes, cells were stained with an isotype control antibody (rabbit polyclonal IgG, abcam, UK; Cat. No.: ab27472 or mouse IgG1, Dako, Denkmark, Cat. No.: X0931).

Statistical analyses

Data were analyzed for a Gaussian distribution and consequently subjected either to parametric tests (t-test or one way ANOVA) or non-parametric tests (Mann-Whitney U Test or Kruskal-Wallis Test) using SPSS software (IBM Version 22).

Results

Effect of CSE on HL-1 cell death under normoxic and hypoxic conditions

To evaluate the effect of CSE on HL-1 cell viability, cells were incubated with freshly made CSE and subjected to our hypoxia and reperfusion model representing MI thus deprivation of nutrients and oxygen. After 24h of CSE incubation and subjection to our hypoxia/reperfusion model, flow cytometry analyses showed significant concentration-dependent increase in apoptotic cells but no effect on the number of necrotic cells under normoxic as well as hypoxic conditions. Hypoxic compared to normoxic conditions further increased

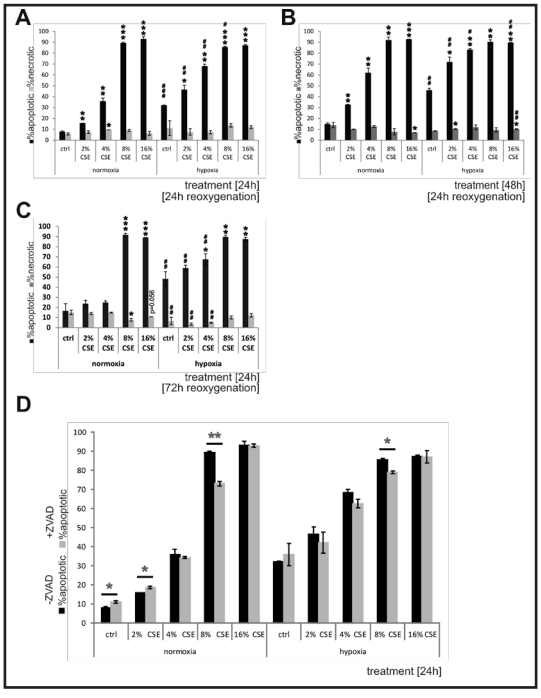

Fig. 2. Effect of CSE on HL-1 cell death under normoxic and hypoxic conditions A, B HL-1 cells were incubated with different concentrations of CSE for the indicated times, subjected to the hypoxia/reperfusion model, stained with Annexin-V/PI and number of apoptotic and necrotic cells was analyzed using flow cytometry C Annexin-V/PI staining after prolonged time for reoxygenation D Annexin-V/PI staining was repeated with addition of ZVAD, a potent inhibitor of caspase-dependent apoptosis. Data expressed as mean ± SD * p<0.05; ** p<0.01; *** p<=0.001; # p<0.05; ## p<0.01; ### p<=0.001.

the number of apoptotic cells (Fig. 2A). This was also observed after 48h of CSE incubation. Moreover, after 48h, hypoxic conditions enhanced the effect of CSE on the viability of HL-1 cells as 2% CSE increased the number of apoptotic cells under normoxic conditions by 18% and by 26% under hypoxic conditions (Fig. 2B). Investigating effects of CSE on HL-1 recovery after hypoxia/reperfusion, time for reperfusion was prolonged to 72h. Further increase of HL-1 cell death was observed (Fig. 2C). Flow cytometry analyses showed that apoptosis was the predominately observed type of cell death; therefore the experiment was repeated with addition of ZVAD to inhibit caspase-dependent apoptosis. A reduction of apoptotic cells was seen only in those incubated with 8% CSE, about 10% under normoxic (p=0.004) and about 5% under hypoxic conditions (p=0.01) (Fig. 2D).

One possible pathway leading to apoptosis is by the activation of P53 [24]. In order to evaluate the involvement of DNA damage in HL-1 cell death Western blot analysis was performed to determine expression of P53. Taking the Ponceau staining into consideration, 24h of CSE incubation led to a concentration-dependent decrease in P53 expression under normoxic conditions. Under hypoxic conditions P53 expression was also reduced by CSE incubation, however, not nicely concentration-dependent. After a longer incubation period, P53 expression slightly increased in those incubated with 4 and 8% under normoxic and in those incubated with 2 and 4% under hypoxic conditions (Fig. 3A and 3B).

Another pathway resulting in apoptosis involves activation of AIF (apoptosis inducing factor) [25]. Immunofluorescence staining was used to investigate its relevance in CSE induced HL-1 cell death (Fig. 3C). No AIF positive cells were observed under normoxic conditions. However, under hypoxic conditions AIF positive cells were found in all groups. About 17% more in those incubated with 2% and about 5% more in those incubated with 4% CSE compared to the control (2% CSE, p=0.004; 4% CSE, p= 0.04). No significant difference was observed comparing incubation with high CSE concentrations to the control (Fig. 3D).

Rapid DNA depletion in necrosis might falsify Annexin-V/Propidium Iodide staining. Therefore release of cellular LDH was determined to further characterize cell death type. After 48h of CSE incubation a significant increase in LDH release was observed in those incubated with 4% and 8% CSE as well as a highly significant increase in those incubated with 16% CSE under normoxic conditions (4% CSE, p=0.003; 8% CSE, p=0.008; 16% CSE, p=<0.001). Under hypoxic conditions a trend for reduction of LDH release in those incubated with lower concentrations of CSE was observed, reaching significance in those incubated with 4% CSE (p=0.001) in comparison to the control. Whereas, LDH release was highly significantly increased in those incubated with 16% CSE (p=<0.001) (Fig. 4A).

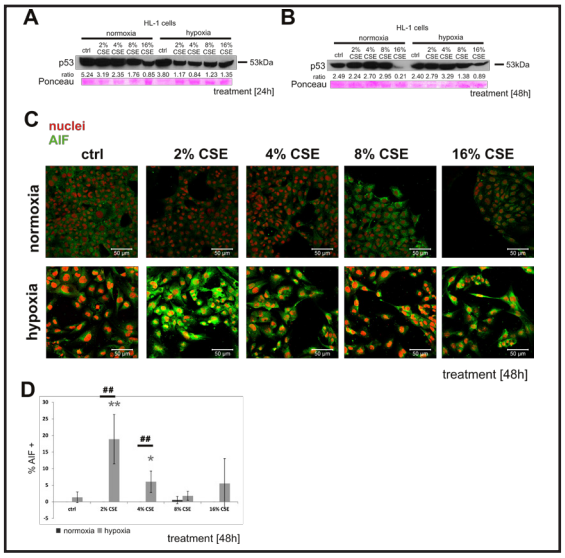

Fig. 3. Analysis of CSE induced cell death mode under normoxic and hypoxic conditions A, B Determination of P53 expression in HL-1 cells after incubation with different concentrations of CSE for indicated times and hypoxia/reperfusion in relation to Ponceau staining C Immunofluorescence staining of HL-1 cells after 48h of CSE incubation and hypoxia/reperfusion with anti-AIF (green); nuclei were stained with PI (red) D Quantification of immunofluorescence staining showing the percentage of AIF positive nuclei after incubation with indicated concentrations of CSE for 48h under normoxic and hypoxic conditions. Data expressed as mean ± SD * p<0.05; ** p<0.01; *** p<=0.001; # p<0.05; ## p<0.01; ### p<=0.001.

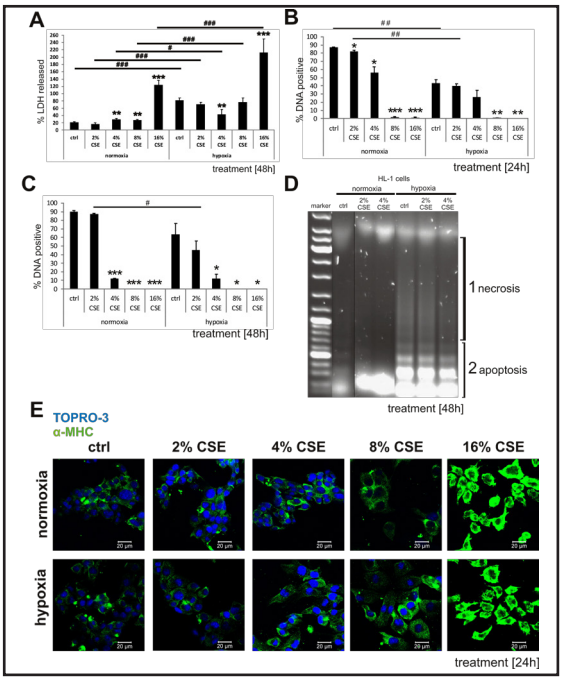

Fig. 4. Analysis of CSE induced cell death mode under normoxic and hypoxic conditions A Quantification of LDH release of HL-1 cells after 48h of CSE incubation and hypoxia/reperfusion B, C Quantification of DNA content of HL-1 cells after 24h and 48h of CSE incubation and hypoxia/reperfusion using PI staining and flow cytometry D Cell-free analysis of DNAse activity of HL-1 cells after 48h of CSE incubation and hypoxia/reperfusion; 1 – indicates location of the smear resembling necrosis, 2 – indicates location of clean cut DNA fragments resembling apoptosis E Immunofluorescence staining of HL-1 cells after 48h of CSE incubation and hypoxia/reperfusion with TOPRO-3 (blue) and anti- α-MHC (green). Data expressed as mean ± SD * p<0.05; ** p<0.01; *** p<=0.001; # p<0.05; ## p<0.01; ### p<=0.001.

To further confirm the hypothesis that higher concentrations of CSE led to rapid necrosis and DNA depletion, intracellular DNA quantities of HL-1 cells after 24 and 48 hours of CSE incubation and subjection to hypoxia/reperfusion were measured. Incubation with CSE for 24h led to a significant concentration-dependent decrease in DNA content under normoxic conditions. Hypoxic conditions further reduced DNA content, but only high concentrations of CSE significantly reduced DNA content compared to the hypoxic control. High concentrations (8% and 16% CSE) led to a complete DNA depletion under normoxic as well as hypoxic conditions (Fig. 4B). This was also observed after 48h of CSE incubation. However, prolonged incubation with 4% CSE under hypoxic conditions also significantly reduced DNA content (Fig. 4C). In order to further assess DNA cleavage, *in-vitro* DNA laddering was performed. Death initiating factors produced under hypoxic conditions led to DNA cleavage in a fashion that indicates an involvement of at the one hand regulated DNAse activity with clear fragment size typical for apoptosis and on the other hand unregulated DNA cleavage resulting in a smear typical for necrosis. Incubation with 2 and 4% CSE led to a decrease of the observed smear under hypoxic conditions.

For further visualization, we performed immunofluorescence staining with TOPRO-3 and anti-α-MHC of HL-1 cells after incubation with CSE and subjection to hypoxia/reperfusion. Under normoxic as well as hypoxic conditions high concentrations of CSE led to loss of cell nuclei, especially in those incubated with 16% of CSE (Fig. 4E).

To determine whether or not the occurring necrosis involves the receptor-interacting-protein-kinase-1 (RIPK-1), which is essential for TNF-induced necrosis [26], the experiment was repeated with addition of Necrostatin-1, an inhibitor of RIPK-1. No biologically relevant effect was observed (Suppl. Fig. I). For all supplemental material see www.karger.com/doi/10.1159/000492285.

Involvement of Oxidative Stress in CSE induced HL-1 cell death

To investigate the involvement of oxidative stress in death of HL-1 cells when incubated with CSE and subjected to hypoxia/reperfusion, staining with 123-Dihydrorhodamine (123-DHR) was performed. After 24h of CSE incubation a trend for dose-dependent reduction in the presence of ROS under normoxic conditions was observed. Presence of ROS under hypoxic conditions was stable but significantly lower than under normoxic conditions (Fig. 5A). After 48h of CSE incubation presence of ROS was stable under normoxic as

well as hypoxic conditions. Presence of ROS was again significantly lower under hypoxic conditions (Fig. 5B). For further investigation of these unexpected results, 123-DHR staining of HL-1 cells subjected to hypoxia and different times of reoxygenation (0h, 1h, 24h, 48h, 72h, 96h) was performed. Hypoxic conditions led to an increase in ROS presence in the first 2 hours of reoxygenation, 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). Addition of N-Acetyl-L-Cysteine (NAC; present during hypoxia and reoxygenation) did not alleviate hypoxia induced cell death (Suppl. Fig. III). A positive control was performed (Suppl. Fig. IV).

To evaluate the influence of the presence of ROS on oxidative modified proteins, OxyBlots after 24h and 48h CSE incubation were performed. Under normoxic conditions after 24h CSE incubation a slight decrease in oxidative modified proteins in those incubated with 2% CSE compared to the control was seen, whereas no differences were observed in higher concentrations, when taking the Ponceau staining of the blot into consideration. Under hypoxic conditions no differences were seen in those incubated with 2%, 4% and 8% CSE compared to the control whereas in those incubated with 16% CSE an increase in oxidative modified proteins were observed, when taking the Ponceau staining of the blot into consideration. Comparing normoxic to hypoxic conditions, in hypoxia less oxidative modified proteins were observed (Fig. 5C). Under normoxic conditions after 48h CSE incubation, no differences were observed in those incubated with 2% or 4% CSE whereas less oxidative modified proteins were seen in those incubated with 8% and 16% CSE compared to the control, when taking the Ponceau staining of the blot into consideration. Under hypoxic conditions no differences were observed in those incubated with 2% or 8% CSE whereas an increase of oxidative modified proteins in those incubated with 4% and 16% CSE compared to the control, when taking the Ponceau staining of the blot into consideration was detectable. Comparing normoxic to hypoxic conditions, in hypoxia less oxidative modified proteins were observable (Fig. 5D).

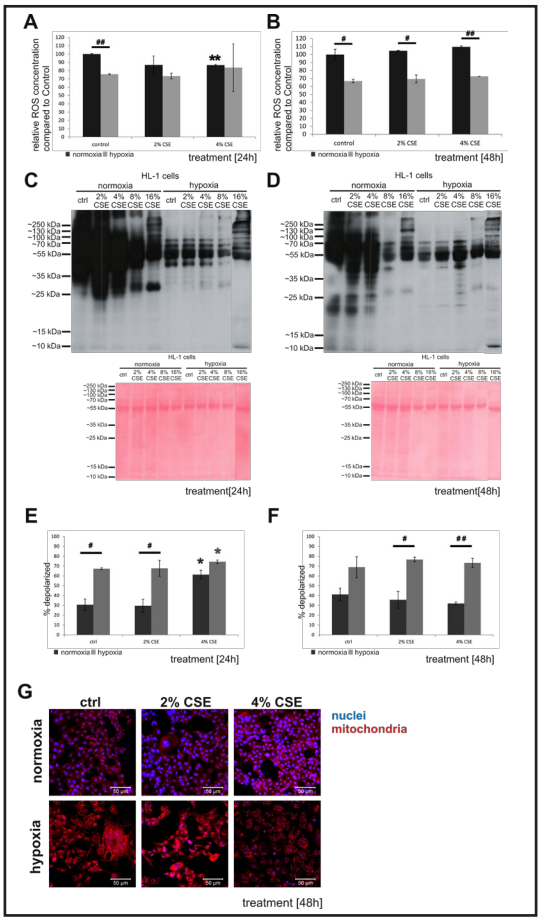

Fig. 5. Influence of oxidative stress on CSE induced HL-1 cell death A, B 123-DHR staining of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion and analysis using flow cytometry. Data collected as median values ± SD and calculated and expressed as percent in relation to normoxic control; # p<0.05 C, D Oxyblot and Ponceau staining of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion E, F JC-1 staining of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion and analysis using flow cytometry. Data expressed as mean ± SD. * p<0.05; ** p<0.01; *** p<=0.001; # p<0.05; ## p<0.01; ### p<=0.001 G Immunofluorescence staining using mitotracker staining and TOPRO-3 (nuclei) of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion.

Involvement of Mitochondria and Lysosomes in CSE induced HL-1 cell death

To elucidate the involvement of mitochondrial depolarization in CSE induced HL-1 cell death JC-1 staining was performed. After 24h, incubation with 4% CSE under normoxic conditions significantly increased the number of cells with depolarized mitochondria by approximately 30% compared to the control (p=0.027) and under hypoxic conditions by about 10% (p=0.04) whereas incubation with 2% CSE had no significant effect. Hypoxic conditions significantly increased the number of cells with depolarized mitochondria in the control group as well as in those incubated with 2% CSE (control, p=0.013; 2% CSE, p=0.037), whereas hypoxia had no additional significant effect on those incubated with 4% CSE (Fig. 5E). After 48h, no significant effect was observed when comparing the control to those incubated with 2% and 4% CSE under normoxic as well as hypoxic conditions. Hypoxic conditions had no significant effect on the depolarization of the mitochondria in the control but significantly increased the number of cells with depolarized mitochondria in those incubated with 2% and 4% CSE by approximately 40% (2% CSE, p=0.022; 4% CSE, p=0.007) (Fig. 5F). To further visualize depolarization of mitochondria, immunofluorescence staining using mitotracker staining after 48h of CSE incubation was performed. Hypoxic conditions led to depolarization of mitochondria; incubation of CSE had no further influence (Fig. 5G).

To evaluate the involvement of lysosomal membrane permeabilization in CSE induced HL-1 cell death, lysosomal labeling was performed. Lysosensor staining was used to identify increased lysosomal pH resembling lysosomal acidification. After 24h CSE incubation, no differences in pH levels were observed comparing those incubated with CSE to the control neither under normoxic nor under hypoxic conditions. Hypoxia showed a trend for less acidification compared to normoxia (Fig. 6A). After 48h CSE incubation, a trend for acidification in those incubated with CSE compared to the control was observed but only under normoxic conditions. Hypoxic conditions induced no changes in pH levels. Again, hypoxia showed a trend for less acidification compared to normoxia (Fig. 6B). Another method for evaluation of LMP namely lysotracker staining was performed to evaluate lysosomal stability. Influence of CSE incubation on lysotracker fluorescence was observed neither under normoxic nor hypoxic conditions after 24h and 48h. Hypoxic conditions led to a statistically relevant decrease in lysotracker fluorescence after 24h and 48h (Fig. 6C and

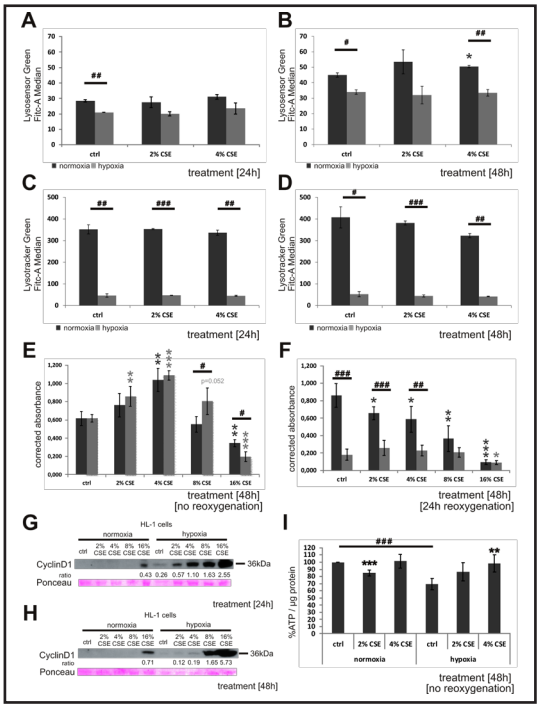

Fig. 6. Effect of CSE on lysosomes and HL-1 proliferation A, B Lysosensor staining of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion and analysis using flow cytometry C, D Flow cytometry based analysis of Lysotracker staining of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion E, F XTT assay of HL-1 cells after 48h of CSE incubation and hypoxia with or without reperfusion G, H Determination of CyclinD1 expression in relation to Ponceau staining of HL-1 cells after indicated times of CSE incubation and hypoxia/reperfusion I Quantification of ATP availability in HL-1 cells after 48h of CSE incubation and hypoxia without reperfusion. Data expressed as mean ± SD. * p<0.05; ** p<0.01; *** p<=0.001; # p<0.05; ## p<0.01; ### p<=0.001.

Fig. 6D).

Effect of CSE on HL-1 metabolic activity under normoxic and hypoxic conditions

When collecting protein samples for Western blot analyses, an increase in the amount of protein with addition of CSE was observed (Suppl. Fig. V). In order to evaluate metabolic activity of HL-1 cells an XTT assay prior to and after reoxygenation was performed. Prior to reoxygenation the incubation with low concentrations of CSE led to a concentration-dependent increase in metabolic activity under normoxic and hypoxic conditions (normoxia: 4% CSE, p=0.003; hypoxia: 2% CSE, p=0.009; 4% CSE, p=<0.001); whereas incubation with higher concentrations showed a trend for a reduction in metabolic activity (normoxia: 16% CSE, p=0.003; hypoxia: 16% CSE, p=<0.001). Comparing normoxic to hypoxic conditions, no differences were found in the control and those incubated with lower CSE concentrations. However, in those incubated with 8% CSE hypoxia led to an increase in metabolic activity but to a decrease in those incubated with 16% CSE (8% CSE, p=0.033; 16% CSE, p=0.01) (Fig. 6E). After reoxygenation, CSE incubation led to a significant concentration-dependent decrease in metabolic activity under normoxic conditions (2% CSE, p=0.038; 4% CSE, p=0.035; 8% CSE, p=0.003; 16% CSE, p=<0.001). Under hypoxic conditions a trend for slightly increased metabolic activity was observed in those incubated with 2%, 4% and 8% CSE and a significant decrease in those incubated with 16%CSE (p=0.033). Hypoxia significantly reduced metabolic activity in the control and those incubated with 2% and 4% CSE; no statistical difference was observed in those incubated with 8% and 16% CSE compared to normoxia (Fig. 6F).

To further assess metabolic activity and proliferation of HL-1 cells when incubated with CSE and subjected to the hypoxia/reoxygenation model, Cyclin-D1 expression, a cell cycle regulatory protein and marker for proliferation, was evaluated using Western blot analysis. Taking the loading control into consideration, after 24h and 48h CSE incubation under normoxic conditions Cyclin-D1 expression was observed only in those incubated with 16% CSE. Under hypoxic conditions a dose dependent increase in Cyclin-D1/Ponceau staining ratio was observed after 24h and 48h, suggesting increased proliferation (Fig. 6G and Fig. 6H).

To evaluate the influence of CSE incubation on ATP availability, the amount of ATP was quantified. Under normoxic conditions 2% CSE led to a highly significant decrease in ATP availability (p=<0.001) whereas no difference in those incubated with 4% CSE compared to the control was observed. Under hypoxic conditions CSE incubation showed a trend of increased ATP availability reaching significance at 4% CSE incubation (p=0.007). Hypoxic conditions only had an effect on the control, highly significantly decreasing ATP availability (p=<0.001) (Fig. 6I).

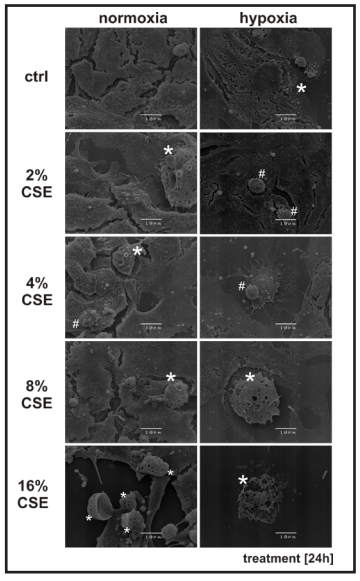

Fig. 7. Scanning electron microscopy of HL-1 cells after 24 hours of CSE incubation (x1500 magnification). HL-1 cells were seeded on glass coverslips, incubated with CSE in the different concentrations (2%, 4%, 8%, 16%) for 24 hours, subjected to the hypoxia/reperfusion model, fixed, dehydrated, desiccated, mounted, sputtered and examined with a scanning electron microscope. * necrotic cell, # apoptotic cell.

Flattening of HL-1 cell shape under hypoxic conditions

Scanning electron microscopy was performed to visualize effects of 24h CSE incubation on HL-1 cell shape under normoxic and hypoxic conditions. Hypoxic conditions resulted in a less three-dimensional structure of HL-1 cells, as they seem to flatten. Concerning the number of viable cells, the observations made via scanning electron microscopy go hand in hand with the results seen

in Annexin-V/Propidium-Iodide staining, whereas the majority of visible dead cells under normoxic conditions can be interpreted as necrotic cells, since characteristic holes in the cell membranes can be seen. However, in a higher magnification we observed a switch from mainly necrotic to apoptotic cells after CSE incubation under hypoxic conditions (Fig. 7).

Discussion

The 'Smoker's Paradox' was postulated by several clinical studies, which paradoxically observed a better outcome of smokers after MI than of non-smokers [9]. Possible underlying mechanisms for this phenomenon remain hypothetical, as there haven't yet been any *in-vitro* studies examining cell biological processes involved. The aim of this *in-vitro* study was to elucidate the effect of CSE, the hydrophilic phase of inhaled cigarette smoke [27], in combination with oxygen and nutrient deprivation following reperfusion simulating MI on the viability of cardiomyocytes. AnnexinV/PI staining showed a dose dependent increase in the amount of dead HL-1 cells after CSE incubation. Adding another stressor, hypoxia and reperfusion further increased cell death. One theory on the 'Smoker's Paradox' states, that cigarette smoke induces pre-conditioning of cardiomyocytes by short episodes of ischemia leading to improved regeneration after hypoxia [28]. However, prolonged reoxygenation did not lead to a recovery of HL-1 cells nor increased viability. Although apoptosis was the main observed type of cell death, inhibition of this type by a common inhibitor, did not generally inhibit cell death neither under normoxic nor under hypoxic conditions (except the reduction of 5% with 8% CSE under hypoxic conditions). As the effect of the inhibitor was inconsistent and not concentration-dependent, caspase-dependent apoptosis does not play a role in CSE induced cell death. Upregulation of P53 is another possible way leading to cell death [24, 25]. Based on our findings, P53 seems primarily not to be involved in CSE induced cell death. However, we observed an increase in P53 expression after a longer incubation period with low-level CSE, suggesting that low concentrations of CSE lead to activation of P53-dependent apoptosis under hypoxic conditions. Aside from caspase-dependent cell death, also caspase-independent cell death is known. One of the main executor proteins is AIF [25, 29]. As we observed a CSE- and hypoxia-dependent AIF activation (in detail the translocation of this protein from the cytoplasm to the nucleus), we conclude the involvement of AIF. At this point and based on this contradictory results we called the results from the Annexin V/PI staining in question and extended our analyses using additional methods. More recent research has shown that cell death cannot be strictly separated in either apoptosis or necrosis. Both may occur in a tissue at the same time. Moreover, a clear verification of cell death mode can only be reached by using an array of different methods. In the Annexin-V/PI staining, the number of necrotic cells remained constant and rather low. However, necrosis can lead to rapid DNA depletion biasing Annexin-V/PI staining [30]. Results of the LDH assay show involvement of necrosis in CSE induced HL-1 cell death and suggest that low-level incubation with CSE leads to a switch from necrosis to apoptosis in HL-1 cells after hypoxia. Measurement of DNA quantities revealed a concentration-dependent decrease in DNA content, which was also seen in immunofluorescence staining arguing for a rapid DNA depletion and the primarily production of false results using Annexin V/PI stainings. Moreover, data observed from the *in-vitro* DNA laddering might indicate a decrease of intracellular necrosis inducing factors of HL-1 cells with low-level CSE incubation. The data is consistent with our findings in the LDH release assay and supports our hypothesis of involvement of necrosis with DNA depletion in CSE induced HL-1 cell death. Of note, also the increase in the amount of AIF-positive cells and the increase in P53 amount under low-concentration CSE treatment in hypoxia argues for the necrosis-apoptosis switch. Addition of Necrostatin-1 had no relevant effect, excluding the RIPK-1 pathway involved in CSE induced HL-1 necrosis [31].

Generation of ROS is known to be involved in several pathological processes e.g. in reperfusion injury. Therefore relevance of oxidative stress in CSE and hypoxia induced cell death was evaluated. Hypoxic conditions led to an increase of oxidative stress, which was not

intensified by CSE incubation. However, no relevant increase in oxidative modified proteins was observed. Therefore, we conclude that oxidative stress is not directly relevant in CSE and hypoxia induced cell death.

The mitochondria of the cell can be thought of as its "powerhouse" and is essential in cell death control as depolarization of the mitochondria is known to be a critical point in cell death – in apoptosis, necrosis and autophagy [26]. Mitochondrial depolarization was not involved in the switch from necrosis to apoptosis, as CSE incubation had no relevant effect on the depolarization of the mitochondria. Recently, it is known that lysosomes play a greater role in cell death than previously thought. Incubation with CSE neither led to an acidification nor to a significant reduction of intact lysosomes. Potentially, this organelle is not involved in CSE/hypoxia induced cell death but it might also be the case that lysotracker staining might not be sensitive enough, as a slight trend of dose-dependent increase in LMP under normoxic and hypoxic conditions can be observed.

The XTT assay revealed increased metabolic activity with the addition of CSE in low concentrations in normoxia and after hypoxia without reoxygenation. Consistent with our understanding of reoxygenation-damage, the viability of HL-1 cells is highly significantly reduced after hypoxia and reoxygenation, but incubation with CSE only further reduced viability in those resembling high-dose-intoxication. Increased metabolic activity combined with dose-dependent increase in CyclinD1 expression and increased protein levels might resemble proliferation of HL-1 cells due to incubation with CSE in the setting of simulated MI. Though, it has to be taken into consideration that an increase in metabolic activity and proliferation could also represent hypertrophy of HL-1 cells as described by Zornoff et al., which in this case has to be considered as a negative effect indeed [32]. However, under the microscope cells incubated with CSE appeared denser and appeared to reach confluence faster, indicating true proliferation.

Various constellations that lead to a necrosis-apoptosis shift have been reported. It is known that ATP availability plays an important role in whether a cell dies the one way or another [33]. Kim et al. showed the influence of ATP generation as determining factor on cell death fate in rat cardiomyocytes [34]. We performed the ATP assay without reoxygenation to evaluate the availability prior to HL-1 cell death, which takes place after reoxygenation shown by the XTT assay. Compared to the control, hypoxia did not reduce ATP availability in those incubated with low-dose CSE, implicating ATP presence as mode of apoptosis-necrosis switch similar as previously described [34, 35]. Moreover, Lim et al. reported a switch from necrosis to apoptosis based on inhibition of ROS production in hepatocellular carcinoma [36]. We observed significant lower presence of ROS in hypoxia unaffected by CSE incubation. Nonetheless, this could be another influencing factor on the necrosis-apoptosis switch in HL-1 cells.

Conclusion

These results, summarized in Fig. 8, suggest an involvement of both - apoptosis and necrosis in CSE induced cell death. The observed trend for less LDH release in hypoxia and reduced necrotic "DNA smear" after low-level CSE incubation might represent a switch from necrosis to an apoptotic death, which might account for the 'Smoker's Paradox' and would explain inconsistent results in clinical studies as the amount of cigarettes smoked has to be taken into consideration. Moreover, we were able to

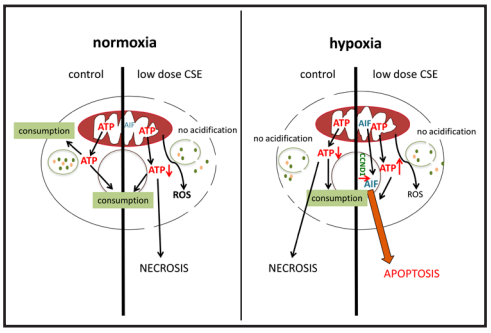

Fig. 8. Schematic representation of the proposed mechanisms of the 'Smoker's Paradox'. Under normoxic conditions low dose CSE incubation leads to oxidative stress, ATP depletion and necrosis of HL-1 cells. Low dose CSE incubation under hypoxic conditions leads to increased CyclinD1 expression and apoptosis via AIF.

show that the necrosis-apoptosis switch is mediated by activation of AIF, increase in P53 and increased ATP availability in combination with an increase in proliferation. Therefore future clinical studies should differentiate between different types of smokers to further investigate the 'Smoker's Paradox'.

Limitations

Obviously, the HL-1 cell line is no primary cell line, but it has been proven to maintain their cardiomyocyte phenotype in culture.

Disclosure Statement

The authors declare to have no competing interests.

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