Cigarette-Smoke- and Age-Dependent Oxidative Stress Effects in Rats *

by

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SUMMARY

Oxidative stress is a basic mechanism involved in both age- and smoking-related diseases. To test whether smoking affects young, old, and calorie-restricted organisms to the same extent, we assessed oxidative stress parameters in the lung, heart, and liver of male Fischer 344 rats (4 months old and 19–22 months old) exposed to air or cigarette mainstream smoke. Smoke-related effects were seen for parameters of DNA damage, lipid peroxidation, protein oxidation, and glycoxidation. No smoke-related effects were observed for DNA damage in the lung and heart (Comet assay) and for malondialdehyde in the lung. The old rats showed higher smoke-related responses than the young rats for 8-hydroxy-desoxyguanosine (8-OHdG) in the heart and liver, DNA damage in the liver, and protein carbonyls in the lung; however, there was little evidence for an overadditive effect of smoking on aging. Caloric restriction, which is known to retard aging effects, also had little impact on smoke-related oxidative changes. [Beitr. Tabakforsch. Int. 26 (2014) 109–120]

ZUSAMMENFASSUNG


RESUME

Le stress oxydatif est un mécanisme fondamental, à l’œuvre tant dans les pathologies liées au tabagisme qu’à celles liées à l’âge. Dans le but de déterminer si le tabagisme affecte, dans une même mesure, les organismes jeunes, vieux et ceux soumis à un régime limité en calories, nous avons
The mechanisms underlying biological aging and the toxicological pathways activated by cigarette smoking are complex and far from fully understood. However, there are several similarities between aging and smoking with regard to their impact on the organism in terms of their final outcomes, i.e., degenerative diseases and increased risk of mortality (1, 2). Two of the basic mechanisms contributing to degenerative diseases and aging, as well as to the effects of smoking, are oxidative stress and glycoxidative stress. Oxidative stress plays an important role in the age-related gradual and progressive loss of physiological function and homeostasis (3, 4). The "oxidative stress theory" (5, 6) states that reactive oxidants generated by the environment or by cellular metabolism increasingly disturb biochemical cellular functions and damage cellular components such as lipids, proteins, and nucleic acids. However, there are also views that question the direction of this causal relationship and postulate that an increased production of reactive oxygen species is simply a manifestation of the cellular disturbances (7, 8).

The major sources of intrinsic reactive oxidants originate from the cellular metabolism itself and, in particular, from the mitochondria (9–11), but other sources of oxidants, such as deposits of lipofuscin in the lysosomes, have also been described in aged cells (12). Conversely, caloric restriction (CR) is known to retard biological aging (13), most likely by reducing endogenous oxidant production (14).

Cigarette smoke contains a large amount of free radicals (15, 16) and compounds interacting specifically with the DNA (17), leading to the known mutagenic and carcinogenic activity of cigarette smoke in humans and experimental systems (18–21). Tobacco and cigarette smoke also contain Maillard reaction products, which result from the reaction of tobacco proteins with tobacco sugars. Similar protein-sugar reactions also occur naturally within the organism, where they can cascade in additional radical stress, resulting in advanced glycation end-products (AGEs). Exogenously absorbed AGEs by nutrition or by cigarette smoke, as a lifestyle factor, pass the receptor for AGEs (RAGE), leading in advanced glycation end-products (AGEs). Exogenously absorbed AGEs by nutrition or by cigarette smoke, as a lifestyle factor, pass the receptor for AGEs (RAGE), which is known to induce additional inflammation processes (22). The complex AGE reactions that lead to glycoxidative stress are involved in the occurrence of several degenerative diseases. Increased levels of AGEs have been found in smokers (23) and AGEs have also been reported to be involved in aging (24).

Because aging is connected with an increased oxidative load, and smoke exposure is an additional source of oxidants, we expected that oxidative stress parameters would be more expressed in old rats than in young rats and more in smoke-exposed rats than in unexposed rats. Of special interest was the question of whether there is an interaction between smoking and aging. We therefore measured a set of established toxicological parameters characterizing oxidative and glycoxidative exposure in the lung and other organs in young and old Fischer 344 rats, either unexposed or exposed to cigarette smoke:

- 8-Hydroxy-deoxyguanosine, as one of the major elimination products of DNA oxidation.
- Comet assay, as a tool to detect general DNA damage such as strand breaks.
- 4-Hydroxynonenal and malondialdehyde, as products of lipid peroxidation.
- Malondialdehyde: a product of oxidative degradation of polyunsaturated lipids.
- Protein carbonyls, as products of protein oxidation.
- Proteasome activity, the activity of an enzyme that degrades damaged proteins, like protein carbonyls by proteolysis.
- Carboxymethyl-lysine, one of the major advanced glycation end-products formed on proteins by combined nonenzymatic glycation and oxidation.
- Glutathione, an endogenous antioxidant.
MATERIAL AND METHODS

Animals and smoke exposure

Young (4 months) and old (19–22 months) male Fischer 344 rats were obtained from the National Institute of Aging, Bethesda, MD, USA. The rats were kept under controlled conditions and allowed to adapt to their new environment for 10 days at Philip Morris Research Laboratories bvba in Leuven, Belgium, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved laboratory (25). The rats (10 per environment for 10 days at Philip Morris Research Lab) were housed in controlled conditions and allowed to adapt to their new environment. The rats were kept under conditions that simulated those of a laboratory setting, allowing them to adapt to their new surroundings.

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Smoke exposure

Smoke exposure was administered to the rats according to BADER et al. (26). The rats were exposed to cigarette smoke for 10 days at Philip Morris Research Laboratories bvba in Leuven, Belgium, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved laboratory (25). The rats were exposed to cigarette smoke for 10 days at Philip Morris Research Laboratories bvba in Leuven, Belgium, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved laboratory (25). The rats were exposed to cigarette smoke for 10 days at Philip Morris Research Laboratories bvba in Leuven, Belgium, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved laboratory (25). The rats were exposed to cigarette smoke for 10 days at Philip Morris Research Laboratories bvba in Leuven, Belgium, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved laboratory (25).

8-Hydroxy-deoxyguanosine (8-OHdG) was determined according to BADER et al. (30). DNA was extracted using the Blood and Cell Culture DNA Kit (Qiagen, Hilden, Germany). The heat-denatured DNA was then analyzed using the Blood and Cell Culture DNA Kit (Qiagen, Hilden, Germany). The heat-denatured DNA was then analyzed using the Blood and Cell Culture DNA Kit (Qiagen, Hilden, Germany).

4-Hydroxynonenal (HNE) was determined according to GIL et al. (32). 2.5 mL of tissue homogenate was incubated for 2 h in the dark with 2.5 mL of dinitrophenylhydrazine solution (1.8 mM in 1 M HCl) at room temperature. The organic phase was extracted with dichloromethane before evaporating it to dryness. The residue was re-dissolved with 1.5 mL of dichloromethane and spotted on thin layer chromatography plates, which were developed with dichloromethane. The zone containing the 4-hydroxynonenal was scraped off, extracted with methanol, and evaporated to dryness. The residue was dissolved in 1 mL of methanol. Methanol/water (4:1, v:v) was used as eluent for the isocratic HPLC analysis.

Malondialdehyde

Malondialdehyde (MDA) was determined according to GIL et al. (32). Tissue samples were boiled in the presence of thiobarbituric acid for 60 min; the reaction was stopped by cooling the samples in an ice bath. After neutralization the samples were analyzed by HPLC using a 150 mm LC-18-S Supelcosil reversed-phase column (Supelco, Deisenhofen, Germany) and isocratic potassium phosphate buffer/methanol as eluent.

Protein carbonyls

Protein carbonyl (PCO) content was determined in the homogenized tissue supernatant with protein concentrations of 2 mg/mL, employing a modified ELISA method (33, 34). An anti-dinitrophenyl rabbit IgG-antiserum (Sigma, Deisenhofen, Germany) was used as the primary antibody and a monoclonal anti-rabbit IgG antibody peroxidase conjugate (Sigma) as the secondary antibody. Development was performed with o-phenylenediamine and H₂O₂.

Proteasome activity

For specific determination of the ATP-independent (20S) proteasome activity (PA), cell lysates were adjusted to 1 mg
protein/mL, and 10 µl of the lysed cells in 3-fold-concentrated incubation buffer was transferred into each well of a 96-well microtiter plate. The fluorogenic peptide suc-LLVY-MCA (Enzo Life Sciences, Plymouth Meeting, PA, USA) was added at a final concentration of 0.2 mM (stock solution 2 mM in DMSO). The mixture was incubated for 30 min at 37 °C. The amount of the cleaved peptide releasing fluorescent MCA is directly proportional to the proteasomal activity. Fluorescence was determined in a fluorometer at an excitation wavelength of 390 nm and an emission wavelength of 460 nm. Free MCA was used as standard. The proteasome-incubation buffer comprised 450 mM Tris-base, pH 8.2; 90 mM potassium chloride; 15 mM Mg-acetate; and 3 mM DTT. The reagents were dissolved in water and stored at $-20 \, ^\circ\mathrm{C}$. The reduction agent DTT was added freshly each day.

Glutathione

Total glutathione was determined by measuring reduced glutathione (GSH) and oxidized glutathione (GSSG) using the Glutathione Assay Kit 703002 (Cayman, Tallinn, Estonia). Basically, the plasma was treated with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB). GSH reacts directly with DTNB, whereas GSSG is reduced by the added glutathione reductase. GSH-TNB derivative was determined at 405 nm.

Carboxymethyl-lysine

For immunohistochemical carboxymethyl-lysine (CML) determination, formalin-fixed paraffin-embedded lung samples were cut and slides were treated with xylol replacement medium XEM-200 (limonene). The samples were boiled and endogenous peroxidase activity was blocked by pretreatment with 1% hydrogen peroxide (35). After washing with PBS, the unspecific reactions of the primary antibody were blocked using goat serum. An anti-CML-rabbit-antibody (gift of Prof. Schleicher, Tübingen, Germany) was used in a 1:2000 dilution. Incubation was performed for 1 h at 37 °C. After washing, cells were incubated at room temperature for 45 min with the biotinylated secondary antibody (anti-rabbit-IgG; Vectastain, Vector Laboratories, Burlingame, CA, USA) in a 1:200 dilution. The Vectastain Elite ABC Peroxidase kit was used for development. Evaluation was done microscopically.

Statistics

All values reported are mean ± standard error (SE). Groups were compared by Student’s $t$-test after exclusion of outliers identified by the Nalimov test. A p-value of < 0.05 without corrections for multiple testing was considered statistically significant.

RESULTS

Table 1 gives an overview of the results obtained. As expected from the literature (3, 36, 37), we found age-related increases in oxidation parameters in our animal model, although the results were not always pronounced. As the lung is the first organ exposed to smoke, we tested oxidative stress parameters in the lung. With regard to

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Table 1. Biomarkers of oxidative stress in old and young rats exposed to air or cigarette mainstream smoke. Units are shown in brackets, AU = arbitrary unit.
DNA damage, an age-related increase in the amount of 8-OHdG in lung tissue was found in the air-exposed rats, and a smoke-related increase was found in young and old rats. A smoke effect was also seen in old rats but it was rather weak and did not reach statistical significance (Table 2, Fig. 1). To test whether this holds true for other organs, we also measured 8-OHdG in the heart and liver. In the heart, age-related increases were indicated in air-exposed rats, and smoke-related increases were seen in both young and old rats, with a very strong increase in old rats (Table 2, Fig. 1). In the liver, there was an age-related decrease and a smoke-related decrease seen in young rats, but a strong smoke-related increase in old rats. Because 8-OHdG is the elimination product of oxidatively damaged DNA, direct measurement of general DNA damage, e.g., DNA strand breaks, should show similar smoke-related features in both age groups. To demonstrate this, we measured DNA damage in the Comet assay, however, neither age- nor smoke-related increases were seen in the lung. Age- and smoke-related increases were indicated in the heart and clearly visible in the liver. In the liver, they were again higher in old rats than in young rats (Table 2, Fig. 2). To further elucidate the age- and smoke-related effects of oxidative stress in the lung, we measured HNE, PCOs, and MDA. An age-related decrease was seen for MDA and PCOs and a smoke-related decrease (not statistically significant) was seen in young rats; while a strong increase was seen in old smoke-exposed rats for PCOs (Table 2, Fig. 3). An age-related

**Figure 1.** Effects of cigarette smoke exposure on the 8-OHdG content in young and old rats. Young rats (4 months old) and old rats (~20 months old) were exposed to fresh air or to cigarette smoke (450 µg TPM/L) for 39 days. Data represent mean ± SE. Statistically significant differences: *a: old compared to young rats, *sy: smoke-exposed compared to air-exposed young rats, *so: smoke-exposed compared to air-exposed old rats (for details see Table 2).
inverse correlation between the PCO content and the 20S proteasome activity has been reported in the lung (38). The expected age-related decrease was observed in smoke-exposed rats but only indicated in unexposed rats. The smoke exposure itself minimally (10%) increased proteasome activity in young but not in old rats (Table 2, Fig. 3).

Because some of the oxidative stress parameters failed to demonstrate the expected increase, we investigated the antioxidative protective capacity as reflected by the total glutathione content. Whereas aging per se was not accompanied by a decline in the glutathione amount in our model but rather by a slight increase, cigarette smoke exposure did lead to a decline of this antioxidative defense capacity in both young and old rats (Table 2, Fig. 4).

To investigate the influence of advanced glycation end-products, we conducted a histochemical investigation of the lung. Age-related increases in air-exposed rats, as well as consistent smoke-related increases in young and old rats, were seen in the alveolar epithelium and lung macrophages for CML (Table 2, Fig. 5).

Figures 2–5: Young rats (4 months old) and old rats (~20 months old) were exposed to fresh air or to cigarette smoke (450 µg TPM/L) for 39 days. Data represent mean ± SE. Statistically significant differences: *a: old compared to young rats, *sy: smoke-exposed compared to air-exposed young rats, *so: smoke-exposed compared to air-exposed old rats (for details see Table 2).
Because caloric restriction has been reported to counteract age-related changes in metabolism and the related oxidant production (39), we also investigated the effects of cigarette smoke in the lungs of old, calorie-restricted rats. Smoke-related increases were seen for DNA-damaging effects, as measured by 8-OHdG and Comet assay; for lipid peroxidation, as measured by MDA; and for the glycoxidative stress parameter CML in lung macrophages; while a decrease was indicated for protein oxidation, as measured by PCO levels. Interestingly, PCO and MDA levels in lung and 8-OHdG in heart of smoke-exposed old rats were reduced by approximately 50% (Table 2, Fig. 6).

**DISCUSSION**

Many of the degenerative diseases and cancers that are associated with aging are seen in both smokers and non-smokers. However, the onset of these diseases generally occurs at an earlier stage of life in smokers than in non-smokers (40–45). The similarities between smoking and aging have been discussed in the literature (46), but have not yet been comprehensively experimentally investigated. With the present work, we aimed to investigate the combined influences of smoke exposure and the complex phenomenon of biological aging.

In order to differentiate between aging and smoking, we needed to first analyze the age-related effects. The age-related increases seen in air-exposed rats, i.e., 8-OHdG in the lung and CML in lung macrophages, support the role of oxidative and glycoxidative stress in aging, while the decreases, i.e., 8-OHdG in the liver and MDA and PCOs in the lung, cannot be explained by oxidative stress. These results might possibly be explained better by the theory that age-related changes induced by oxidative stress may be balanced to a certain degree by an organism’s intrinsic defense metabolism (47, 48). No age-related changes were seen for the other parameters in this model, which appears to contradict the theory that oxidative stress induces aging directly. However, there is an abundance of contradictory results in the literature for the same parameter in the same tissue from different laboratories, which may be explained by differences in species, strain, sex, and age of the rats investigated (49–55). Moreover, such inconsistencies have been reported from the same laboratories either with the same parameter in different tissues (56–58) or with different parameters related to oxidative stress in the same tissue (59–62). One important supporting finding was published by JANA et al. (63), who found species-specific differences in the age-dependent carbonylation of different proteins from different tissues within an organ and concluded that age-dependent carbonylation is a highly selective phenomenon rather than a randomized process, such as those generally associated with aging. It cannot be excluded that such specification may also be true for the other parameters. We randomized the organ tissue using the technique proposed by NYENGAARD (29), which does not allow differentiation between different tissues within an organ.
because we wanted to ensure that we obtained a general result for each organ as a whole, and because it is not known which tissues within an organ respond to aging and oxidative stress. However, we cannot exclude the possibility that the sensitivity of these toxicological parameters is not sufficient to consistently show an overall age-related increase in damage, which may be more subtle than a toxic insult.

We analyzed smoke-related effects in young rats, which were 6 months old and sexually mature. This is different from the majority of smoke-related investigations, which generally use much younger rats, i.e., 8- to 10-week-old rats as suggested in OECD guidelines (64) and generally practiced in tobacco product testing (28). We chose to use 6-month-old rats to minimize the likelihood of interference from maturation processes. The smoke-related increase seen in the lung and heart (8-OHdG) and liver (Comet assay) support the role of oxidative stress in smoking, while the increase in CML in lung macrophages and alveolar epithelium supports the role of glyoxidative stress in smoking. Proteasome activity in the lung indicates increased elimination of damaged proteins.

The subchronic exposure regimen was chosen for this experiment to limit the possibility that the organism may adapt to the smoke (65); therefore, we expected to see a smoke effect on all of the oxidative stress parameters investigated. The decrease seen in glutathione in plasma indicates that the oxidative stress associated with smoke inhalation leads to an increased consumption of this antioxidant. However, the remaining antioxidant capacity might still represent an effective defense system, which may have counteracted effects in the other oxidative stress parameters. Depletion of glutathione by cigarette smoke has been described (66). The decrease in 8-OHdG in the liver may be due to the detoxification of cigarette smoke toxins by the xenobiotic metabolism (67) and the higher elimination of 8-OHdG due to nuclease activity (68). Again, different results to literature may be due, to some extent, to differences in sex, age, and species of the models investigated, however, they may also be due to differences in the smoke exposure conditions.

Analyzing the biomarkers in terms of the combined influence of smoke exposure and biological aging, which has not been investigated in this model before, revealed no clear pattern of effects. In fact, effects were not always more pronounced in old rats. This underlines the complexity of both the mechanisms of aging and the toxicity of cigarette smoke. With regard to DNA damage, the increases seen in older rats in the heart (8-OHdG) and the liver (8-OHdG and Comet assay) suggest that the older rats are more susceptible to the cigarette smoke toxins that reach the circulatory system. No increase in DNA strand breaks was seen with the Comet assay in the lung, even though 8-OHdG showed both an age-related increase and a smoke-related increase. However, the time between last exposure and dissection might have been too long. It has been reported, although under rather different smoke exposure conditions (1-min exposure and extremely high smoke concentration), that the level of DNA strand breaks in the lung returns to control values already after 1 hour (69). In vitro, cigarette smoke is clearly active (70). In peripheral lymphocytes of smoke-exposed rats the assay shows, at best, a weak response (71). The failure of the Comet assay in our study to detect any increase in the lung is possibly due to the cytotoxicity-related loss of damaged cells due to the relatively high mainstream smoke exposure concentration (72). We could identify only two cigarette smoke inhalation studies in the literature where the Comet assay was applied to lung tissue. Both studies found a positive effect, however, despite the high smoke concentrations (dilution around 1/7, in our study 1/100), the short daily exposure times (10 min and 1 min, respectively) resulted in lower daily inhalation doses (time × concentration). Theoretical doses were 75% and 10%, respectively (73, 69); however, it can be assumed that the actual doses were significantly lower, as rodents, if briefly exposed, are known to lower their respiratory minute volume drastically as a function of the irritative potency of the inhaling (74).

A further explanation for the failure of the Comet assay to show smoke-related responses in our study could be the cross-linking effect of aldehydes in smoke, which can inhibit DNA migration preventing the detection of increases in strand breaks in the assay (72, 75). It is also known that some smoke constituents are more readily metabolized or metabolized differently in the lung than other smoke constituents (76–78). There are modifications of the Comet assay reported that might have improved the ability to detect DNA damages in our system, especially those induced by reactive oxygen species, including those via 8-OHdG formation (79, 80). A decrease in liver and lung 8-OHdG levels was observed in rats exposed to benzo[a]pyrene (a proxy for the polycyclic aromatic hydrocarbons in cigarette smoke), but this was accompanied by increased levels in bulky DNA adducts in these organs (81). The only parameters where the combined effects of smoke exposure and biological aging are greater than both the age-related increases alone and the smoke-related increases alone are DNA damage in the heart (8-OHdG) and in the liver (Comet assay). However, considering the inherent variability of the assays, these data are compatible with the effects being even less than additive.

The strong increase in PCO seen in the lungs of old rats, though expected (38, 82), is somewhat surprising in light of the fact that both a smoke-related decrease in the young rats and an age-related decrease in air-exposed rats were seen. These decreases may be due to the organism’s intrinsic defense metabolism, e.g., detoxification, or to the higher elimination of the damaged proteins, which may, in turn, be due to the higher smoke-related proteasome activity (83) observed in our study, or to the specificity of carbonylation of different proteins (84), as mentioned above. However, in the context of aging combined with smoking, a threshold may be exceeded which can lead to the deactivation of defense proteins, enabling even greater increases in damaged proteins, as indicated in smoke-exposed mice with a deficiency in one of the defense proteins (62). This deactivation of defense proteins remains to be further investigated in the context of smoke exposure.

Old calorie-restricted rats were used to test whether the expected reduction of oxidative burden due to caloric restriction (85) is reflected by a better resistance to cigarette smoke. However, for some parameters in the lung and lung macrophages (Comet assay, MDA, and CML), the calorie-restricted rats appeared to be more vulnerable to smoke exposure.

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exposure. We speculate that this may be the result of reduced antioxidative repair efficiency, i.e., that the calorie-restricted organism adapts to the lack of endogenous stress by economically reducing its defense mechanisms. By adapting in this way, the organism may no longer be able to cope efficiently with a sudden load of oxidative stress, such as subchronic cigarette smoke exposure. As it is know that adaptation may play a major role, a lifetime study could help to clarify the combined impact of caloric restriction; however, the direction of the responses might very well depend on the balance between the degree of caloric restriction and the strength of the induced oxidative stress. Within one publication, Vučević et al. (86) reported a decrease in MDA and an increase in superoxide dismutase due to ethanol-induced oxidative stress after caloric restriction to 60 to 70% of ad libitum intake, but an effect in the opposite direction after restriction to 40–50%.

CONCLUSION

With our experimental approach, we found evidence that the oxidative stress caused by aging and by smoking resulted in some common changes for several of the parameters tested. However, there is little evidence of synergistic effects. In order to obtain a more detailed understanding of the connection between aging and smoking and the possible acceleration of aging by cigarette smoke, future experiments should include the determination of dose- and time-dependency for both aging and smoking, large-scale data sets (omics) and network-based approaches (87) and related approaches as discussed in “21st Century Toxicology” (88–91) may be worth pursuing.

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