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Research Paper

Lipid mediators involved in the oxidative stress and antioxidant defence of human lung cancer cells

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ABSTRACT

Background: The oxidative modifications of bioactive macromolecules have important roles in carcinogenesis. Of particular interest are lipid peroxidation products, which are involved in the activation of Nrf2 and endocannabinoids that affect cancer progression.

Methods: In lung cancer tissues (squamous cell lung carcinoma - SCC and adenocarcinoma - AC), the glutathione peroxidase and catalase activity and glutathione level, together with the expression of Nrf2 and its activators/inhibitors were estimated. The oxidative modifications of DNA (8-hydroxy-2′-deoxyguanosine and *N7*-methylguanine), endocannabinoids (anandamide and 2- arachidonylglyceriol), their receptors (CB1/2, TRV1, GPR55), phospholipid fatty acids (arachidonic, linoleic and docosahexaenoic), and reactive aldehydes (4-hydroxynonenal, 4-oxononenal and malondialdehyde) were determined.

Results: Tumour tissues showed lower antioxidant capacity than healthy tissues, which was accompanied by lower levels of fatty acids and higher levels of reactive aldehydes. Disturbances in antioxidant capacity and enhanced DNA oxidative modifications were observed in 88% of AC patients and 81% of SCC patients. The 4-hydroxynonenal-Histidine adducts were detected in the necrotic and stromal cells in all tumours. These findings were associated with the enhanced Nrf2 activity, especially in AC. The strong difference between the cancer subtypes was evident in the levels of endocannabinoids, with an increase in 89% of SCC and a decrease in 85% of AC patients being observed. Additionally, the increase in the expression of CB1/2 receptors was observed only in 82% of AC, while the expression of VR1 and GPR55 was enhanced in 79% of SCC and 82% of AC patients.

Conclusions: This study shows significant differences in the redox status, Nrf2 pathway and endocannabinoid system between SCC and AC tissues. Understanding the relation between the various lipid mediators and antioxidants in different lung cancer subtypes may be beginning for further research on the effective anticancer therapy.

Abbreviations: 2-AG, 2-arachidonylglyceriol; 4-HNE, 4-hydroxynonenal; 4-ONE, 4-oxononenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AA, arachidonic acid; AC, adenocarcinoma; AEA, anandamide; AKT, serine/threonine-specific protein kinase; ALE, advanced lipoxidation-end products; ARE, antioxidant response element; Bach1, BTB and CNC homology 1, basic leucine zipper transcription factor; CAT, catalase; CB1/2, cannabinoid receptor type ½; COX-2, cyclooxygenase 2; Cul3, cullin-3; DGR, double glycine repeat domain; DHA, docosahexaenoic acid; EGFR, epidermal growth factor receptor; ERK1/2, mitogen-activated protein kinase 3/1; ESI, electrospray ionization; FAAH, fatty acid amide hydrolase; FAME, fatty acid methyl ester; GPR55, G protein-coupled receptor 55; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; HO-1, heme oxygenase 1; JNK1/2, c-Jun N-terminal kinases ½; KAP1, KRAB-associated protein-1; Keap1, kelch-like associated protein 1; LA, linoleic acid; LC-MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MAGL, monoacylglycerol lipase; MDA, malondialdehyde; *N7*-MeG, *N7*-methylguanine; NFκBp52, nuclear factor kappa-light-chain-enhancer of activated B cells, subunit p52; Nrf 1/2/3, nuclear factor erythroid-2 related factor 1/2/3; NSCLC, non-small cell lung cancer; p21, protein 21; p62, protein 62; PBS, phosphate-buffered saline; PUFA, polyunsatutated fatty acids; ROS, reactive oxygen species; SCC, squamous cell lung carcinoma; TBST, tris-buffered saline and tween 20; TIMP-1, tissue inhibitor of matrix metalloproteinases-1; TRV1, transient receptor potential cation channel subfamily V member 1; UPLC-MS/MS, ultra-performance liquid chromatography, tandem mass-spectrometry

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1. Introduction

Lung malignancies are the leading cause of death in both men and women. More than 85% of lung cancers are non-small cell lung cancers (NSCLC), including the two most common histological subtypes of NSCLC: squamous cell lung carcinoma (SCC – approximately 30% of all lung cancers), and adenocarcinoma (AC – approximately 40% of all lung cancers, occurring more often in women than in men and in younger people than other subtypes of lung cancers) [1].

Cancer development is characterised by redox imbalance with a shift towards oxidative conditions. As a consequence, the oxidative modifications of cellular components, including phospholipids, DNA and proteins, are accumulated in tumour cells causing additional disturbances in their metabolism [2]. Several studies have shown that the lipid peroxidation products such as reactive aldehydes are involved in the intracellular signalling pathways and activation of transcription factors in cancer cells [3].

Nrf2 (nuclear factor erythroid-2 related factor 2) is one of the major transcription factors that are constitutively activated in many cancer cells [4]. Nrf2 is a cap 'n' collar basic leucine zipper transcription factor, which regulates the expression of antioxidant enzymes and several anti-apoptotic proteins, which confer cytoprotection against oxidative stress and apoptosis [5]. Nrf2 activates the transcription of target genes through binding to the antioxidant response element (ARE) found in those gene promoters. The genes regulated by Nrf2 encode antioxidants, xenobiotic metabolism enzymes and several ATP-dependent drug efflux pumps [6]. Therefore, the constitutive activation of Nrf2 in cancer promotes tumourigenicity and contributes to chemoresistance. However, the xenobiotic metabolism enzymes in conjunction with drug efflux proteins detoxify cancer drugs, whereas antioxidants provide cytoprotection by attenuating drug-induced oxidative stress and apoptosis [7]. Therefore, chemotherapy for advanced, inoperable NSCLC is generally palliative.

Nrf2 activity is regulated by its cytoplasmic anchor – Keap1 (Kelch-like ECHassociated protein 1) that forms a complex with the transcription factor. Several studies have shown that the constitutive activation of Nrf2 in lung cancer is strongly connected with mutations in the *Keap1* gene [8,9]. In physiological conditions, Keap1 constitutively suppresses Nrf2 activity; however, electrophilic compounds hamper the Keap1-mediated proteasomal degradation of Nrf2, leading to the transcriptional induction of target genes that ensure cell survival [10]. It was found that in the case of lung cancer, mutation of a glycine to cysteine in its DGR domain reduces the affinity of Keap1 for Nrf2 [11]. It was found that Keap1 is a cysteine-rich protein that is highly susceptible to electrophilic molecules, such as reactive oxygen species (ROS) or reactive aldehydes, which are the products of lipid peroxidation (e.g., 4-HNE). Their interactions with Keap1 lead to changes in its confirmation and thereby to Nrf2 activation. Complexes of 4-HNE-Keap1 were detected in NSCLC [12]. It was also observed that 4-HNE occurs at a higher level in human lung cancer cells and acts as a signalling molecule promoting tumour cell viability [13]. Other lipid mediators, such as endocannabinoids like anandamide (AEA) and 2-arachidonylglyceriol (2-AG), are also known to affect the progression of cancer development [14], but the exact mechanism of their action is unknown. Endocannabinoids belong to the endocannabinoid system, which is involved in the reaction of cancer cells to the generation of higher levels of reactive oxygen species [15]. Moreover, natural and synthetic cannabinoids as well as their receptors, CB1, CB2, TRV1, and GPR55, and enzymes involved in the endocannabinoid metabolism have been reported to affect cancer growth at more than one step in several subtypes of malignancies [16]. Accordingly, previous studies identified the anti-tumourigenic activities of cannabinoids, such as inhibition of tumour cell proliferation [17] and angiogenesis [18], as well as induction of apoptosis and autophagy [19]. Cannabinoids and endocannabinoid-related compounds were able to affect lung cancer cell proliferation, induce apoptosis, and inhibit migration and invasiveness [20]. In NSCLC, cannabinoids inhibit cancer cell invasion *via* increasing the expression of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) [21]. It was shown that the endocannabinoid system components protect cancer cells against the higher levels of reactive oxygen species [15] and affect Nrf2 activity [22].

Therefore, the aim of this study was to evaluate the relationship between the lipid mediators and activity of the transcription factor and compare these relationships in the two main subtypes of human NSCLC: squamous cell lung and adenocarcinoma. Finding and understanding the differences in the mechanisms of Nrf2 activation in these cancers may be applied in anticancer therapies.

2. Material and methods

Surgically resected non-small cell lung cancer [NSCLC] and adjacent normal tissue specimens were collected from a group of 28 female and 44 men with a mean age of 62 (46 – 77) years. All patients underwent pulmonary resection for primary NSCLC in the Department of Thoracic Surgery of the Medical University of Bialystok, Poland and the University of Zagreb School of Medicine, Clinical Hospital Centre Zagreb, Division of Pathology, Croatia. Thirty eight samples from patients with pulmonary squamous cell carcinoma (SCC) (12 female and 26 men with mean age of 62 ((46 – 77)years)) and 34 samples from patients with adenocarcinoma (AC) (16 female and 18 men with mean age of 60 ((50 – 72) years)) were collected.

The inclusion criteria for the current study were the following: original diagnosis of lung AC or SCC based on the histologic evidence of glandular differentiation or squamous differentiation, respectively; *completely resected tumour (free resection margins);* stage I or stage II NSCLC; a minimum of three-year follow-up including monitoring for events of cancer recurrence and lung cancer–related death; availability of representative fresh-frozen tumour specimens (the material containing at least 50% tumour cells for DNA extraction); and no adjuvant chemotherapy. This study was approved by the Institutional Ethical Committee of the Medical University of Bialystok as well as the University of Zagreb School of Medicine and informed consent was obtained from each patient. The exclusion criteria were as follows: lack of written consent; recent treatment with certain medications, including nonsteroidal anti-inflammatory drugs, steroids, and oral contraceptives; alcohol abuse; and heavy smokers.

A piece of each collected tissue was homogenised under standardized conditions; 10% homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatants were stored at –80 °C and used for the estimation of biochemical parameters.

2.1. Antioxidant parameters

2.1.1. Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px – EC.1.11.1.6) activity was assessed spectrophotometrically using the method of Paglia and Valentine [23]. GSH-Px activity was assayed by measuring the conversion of NADPH to NADP. One unit of GSH-Px activity was defined as the amount of enzyme catalysing the oxidation of 1 μmol NADPH min^{-1} at 25 °C and pH 7.4. Enzyme specific activity was expressed in units per mg of protein.

2.1.2. Determination of catalase activity

Catalase (CAT – EC.1.11.1.9) activity was determined by

measuring the decrease in hydrogen peroxide absorbance at 240 nm [24]. One unit of CAT was defined as the amount of the enzyme required to catalyse the decomposition of 1 μmol hydrogen peroxide to water and oxygen for 1 min. Enzyme specific activity was expressed in units per mg of protein.

2.1.3. Determination of glutathione level

Glutathione and its oxidised form were quantified using capillary electrophoresis (CE).method of Maeso [25]. Samples were sonificated in the Eppendorf tubes with 2 ml of a mixture containing AcN/H2O (62.5:37.5, v/v) and centrifuged at 29,620 g for 10 min. The supernatant was immediately measured by CE. The separation was performed on a capillary with 47 cm total length (40 cm effective length) and 50 m i.d. and was operated at 27 kV with UV detection at 200 ± 10 nm. The GSH concentration was determined using a calibration curve range: 1 − 120 nmol/L (r2-0.9985).

2.2. Nrf2/ARE pathway

Western blot analysis of cell protein was performed according to Eissa and Seada [26]. Whole homogenate were mixed with Laemmle buffer containing 5% 2-mercaptoethanol, heated at 95 °C for 10 min, and separated by 10% Tris-Glycine SDS-PAGE. Separated proteins were electrophoretically transferred into nitrocellulose membrane. The blotted membrane was blocked with 5% skim milk in TBS-T buffer (5% Tween 20) for 1 h. Primary monoclonal antibodies were raised against Nrf2, phospho-Nrf2 (pSer40), Nrf3, p62, Keap1, ERK1/2, phospho-ERK1/2 (pThr202/pTyr204), cJun, phosphor-cJun (pSer63), NFκB (p52), (Santa Cruse Biotechnology, Santa Cruz, CA, USA), Nrf1, Bach1, HO-1 (Sigma-Aldrich, St. Louis, MO, USA), p21, and KAP1 (Abcam Inc., Cambridge, MA, USA) were used at a concentration of 1:1000. Protein bands were visualised using the BCIP/NBT Liquid substrate system (Sigma-Aldrich, St. Louis, MO, USA) and were quantitated using the Versa Doc System and Quantity One software (Bio-Rad Laboratories Inc., CA). Total protein content in homogenate was measured using a Bradford assay [27].

2.3. DNA modifications

2.3.1. Determination of 8-hydroxy-2′-deoxyguanosine level

8-hydroxy-2′-deoxyguanosine (8-OHdG) was assayed by modified LC-MS method of Dizdaroglu et al. [28]. DNA isolation was performed using Sigma's GenElute Mammalian Genomic DNA Miniprep kit. DNA concentration in the preparations was determined spectrophotometrically. DNA samples were stored at −70 oC until hydrolysis. DNA hydrolysis to nucleosides: DNA samples (200 μl) were mixed with 100 μl of 40 mM sodium acetate, 0.1 mM ZnCl2, pH 5.1 and 20 μl nuclease P1 solution (20 μg protein). Samples were incubated for one hour at 37 oC. Thereafter 30 μl of 1 M Tris–HCl, pH 7.4 and 5 μl of alkaline phosphatase solution containing 1.5 units of the enzyme were added to each sample following 1 h incubation at 37 °C. All DNA hydrolysates were ultrafiltered using Ultrafree-MC filter units (cut off 5000 Da). 8-OHdG in hydrolysates was determined using Agilent 1290 LC system and Agilent 6460 triple quadrupole mass spectrometer equipped with electrospray ionization ESI source. Solvent A [0.1% formic acid in water] and solvent B [0.1% formic acid in methanol] were used in gradient mode to achieve the desired sample separation. The flow rate was 0.4 ml/min. The following gradient was run: 0 min, 5% solvent B; 0–8.0 min, 50% solvent B; 8.0–8.1 min, 100% solvent B; 8.01–12.0 min, 100% solvent B; 12.0–13.0 min, 5% solvent B. LC–MS/MS analysis was performed using a Agilent 1290 HPLC system interfaced with a Agilent 6560 triple quadrupole mass spectrometer with electrospray ion source (ESI). The samples were analysed in the positive ion multiple reaction monitoring (MRM) mode and the transitions of the precursors to the product ions were as follows: *m/z* 284.1 → 168 (quantifier ion) and 284.1 → 69 (qualifier ion). The calibration curve for detection of 8-OHdG was linear in the study range of 50–500 fmol. The LOQ and LOD was 3.0 and 1.5 residues/106 dG. The amount of 8-OHdG was calculated as the number per 105 deoxyguanosine.

2.3.2. Determination of N7-methylguanine level

N7-methylguanine (*N7*-MeG) was assayed by modified LC-MS method of Chao et al. [29]. The DNA solutions were subjected to neutral thermal hydrolysis at 100°C for 30 min. The DNA was precipitated and the supernatant was dried under vacuum and redissolved in 200 ml of 96% (v/v) acetonitrile with 0.1% formic acid for HPLC analysis. LC–MS/MS analysis was performed using a Agilent 1290 HPLC system interfaced with a Agilent 6560 triple quadrupole mass spectrometer with electrospray ion source (ESI). The samples were analysed in the positive ion multiple reaction monitoring (MRM) mode and the transitions of the precursors to the product ions were as follows: *m/z* 166 → 149 (quantifier ion) and 166 → 124 (qualifier ion) for *N7*-MeG, *m/z* 171 → 153. Linear calibration curve covering the range 1– 10,000 pg was obtained by serial dilution of calibration solution. The LOQ was determined to be 0.06, ng/ml on-column. The LOD, defined as the lowest concentration that gave a signal-to noise ratio of at least 3, was found to be 0.02 ng/ml on-column (6.1 fmol, which correspond to 1.6 adducts per 106 unmodified bases).

2.4. PUFAs and lipid peroxidation products

2.4.1. Determination of PUFAs level

Arachidonic (AA), linoleic (LA) and docosahexaenoic (DHA) acids were determined by gas chromatography [30]. Lipid components were isolated from cell lysates by extraction using chloroform/methanol mixture (2:1, v/v). Using TLC, total phospholipids were separated with the mobile phase heptane - diisopropyl ether – acetic acid (60:40:3, v/v/v). All lipid fractions were transmethylated to fatty acid methyl esters (FAMEs) with boron trifluoride in methanol reagent under nitrogen atmosphere without previous separation from the layer. The FAMEs were analysed by gas chromatography with a flame ionisation detector. Separation of FAME was carried out on capillary column coated with Varian CP-Sil88 stationary phase.

2.4.2. Determination of lipid modifications

Lipid peroxidation was estimated by measuring the level of 4-hydroxynonenal (4- HNE), 4-oxononenal (4-ONE) and malondialdelhyde (MDA). Aldehydes were measured by GC/MSMS, as the *O*-PFB-oxime-TMS derivatives, using modified method of Luo [31]. Benzaldehyde-D6 as an internal standard was added to the cell lysates and aldehydes were derivatized by the addition of *O*-(2,3,4,5,6-pentafluoro-benzyl) hydroxyamine hydrochloride (0.05 M in PIPES buffer, 200 μl) and incubating for 60 min at room temperature. After incubation, samples were deproteinized by the addition of 1 ml of methanol and *O*-PFBoxime aldehyde derivatives were extracted by the addition of 2 ml of hexane. The top hexane layer was transferred into borosilicate tubes, and evaporated under a stream of argon gas followed by the addition of *N,O*-bis(trimethylsilyl)trifluoroacetamide in 1% trimethylchlorosilane. A 1 μl aliquot was injected on the column. Derivatized aldehydes were analysed using a 7890 A GC – 7000 quadrupole MS/MS (Agilent Technologies, Palo Alto, CA) equipped with a HP-5 ms capillary column (0.25 mm internal diameter, 0.25 μm film thickness, 30 m length). Derivatized aldehydes were detected in selected ion monitoring (SIM) mode. The ions used were: *m/z* 332,0 and 181,0 for 4-ONE-PFB, *m/z* 333.0 and 181.0 for 4-HNE-PFB-TMS,

m/z 204,0 and 178,0 for MDA-PFB and *m/z* 307.0 for IS derivatives.

2.4.3. Determination of 4-HNE-protein adducts

The 4-HNE-protein adduct ELISA is a method for the detection of 4-HNE bound to proteins, which is considered as the most likely form of 4-HNE occurrence in living systems. This ELISA method was utilised from protocol described by Weber et al. [32] that was adapted from original method by Borovic et al. [33]. 4-HNE-BSA standards in the range from 0 to 250 pmol/mg were used, with final concentration of BSA being 10 mg/ml. Accordingly, all plasma samples were diluted in PBS to protein concentration of 10 mg/ml. Protein concentration of standards and plasma was determined by the BioRad assay according to Bradford [37]. Prepared standards/plasma samples were diluted 10-fold in 50 mM carbonate binding buffer (pH 9.6; 15 mM sodium carbonate, 35 mM sodium bicarbonate) into ELISA plate wells (Nunc Immuno Maxisorp, Thermo Scientific) for duplicate analysis in 100 μl volumes per well. Proteins were adsorbed for 5 h at 4°C. Plates were washed once with PBS and incubated with blocking solution (5% fat free dry milk in carbonate binding buffer) for 2.5 h at room temperature (RT) followed by one washing step (0.1% Tween 20 in PBS), all done in 300 μl per well volumes. 100 μl of primary antibody solution in 1% BSA in PBS was incubated at 4°C overnight (genuine anti 4-HNE-His murine monoclonal antibody, clone 4- HNE 1g4; 1:100, v/v). After washing the wells seven times (0,1% Tween 20 in PBS), the plates were incubated for 30 min with peroxidase blocking solution (3% H2O2, 3% fat free dry milk in PBS) at RT, followed by seven times washing step. 100 μl of the goat anti-mouse secondary antibody solution in 1% BSA in PBS (1:100; Dako, Carpinteria, CA, USA) was incubated for 1 h at RT, followed again by a seven time washing step. 100 μl of chromogen substrate solution (0.1 mg/ml TMB, 0.012% H2O2) in citric buffer (26.5 mM citric acid anhydrous, 51 mM sodium hydrogen phosphate dihydrate) was incubated for 40 min at RT. The reaction was stopped by adding 50 μl of 2 M sulphuric acid. Absorption was read at 450 nm with the reference filter set to 620 nm. Amounts of 4-HNE–protein adducts measured by the ELISA were expressed as pmol 4-HNE per mg of proteins.

4-HNE-His immunohistochemistry of the tissue specimens was done using the same monoclonal antibodies, as was done for the 4-HNE-ELISA. For the immunohistochemical detection of the HNE-adducts the immunoperoxidase technique was used, with secondary rabbit-anti-mouse antibodies (Dako, Glostrup, Denmark), applied on 5 μm sections of the formalin-fixed paraffin embedded sections of the tumours, as described before [34].

2.5. Endocannabinoid system

2.5.1. Determination of endocannabinoids level

Anandamide (AEA) and 2-arachidonylglyceriol (2-AG) were quantified using modified ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) by Lam method [35]. Octadeuterated endocannabinoids: AEA-d8 and 2-AG-d8 as internal standards were added into the homogenate and all cannabinoids were isolated using a solid phase extraction (SPE). UPLC–MS/MS analysis was performed using an Agilent 1290 UPLC system with a Zorbax Extend C18 column (2.1 mm × 150 mm, 1.8 mm, Agilent, Santa Clara, CA, USA) and interfaced with an Agilent 6460 triple quadrupole mass spectrometer with electrospray ionisation source (ESI). The samples were analysed in positive-ion mode using multiple reaction monitoring (MRM). Transition of the precursor to the product ion for AEA was: *m/z* 348.3→62.1, and for 2-AG: *m/z* 379.3→287.2. The linear dynamic range was 0.2–200 ng/g for AEA and 0.8–100 μg/g for 2-AG. The LOQ value was 0.2 ng/g for AEA, and 0.8 μg/g for 2-AG, while the LOD values were 0.02 ng/g and 0.08 μg/g, respectively.

2.5.2. Determination of MAGL activity

Monoacylglycerol lipase (MAGL - EC 3.1.1.23) activity was measured in the homogenate prepared in 20 mM Tris, 320 mM sucrose, 1 mM EDTA, pH 8.0. Supernatant was obtained from the soluble fraction after spinning the homogenate at 1000 g for 15 min. Reaction mixture containing liver supernatants, 10 mM Tris, 1 mM EDTA pH 7.2 was preincubated at 4 °C for 15 min. After addition of arachidonoyl-1-thio-glycerol (A-1-TG) mixture was incubated at 37 °C for 5 min and after cooling to room temperature 1 mM DTNB was added. After 3 min hydrolysis the absorbance at 412 nm was measured. Enzyme specific activity was expressed in nmoles of TNB releasing during 1 min per mg of protein [36].

2.5.3. Determination of FAAH activity

Fatty acid amide hydrolase (FAAH - EC 3.5.1.99) activity was measured in the homogenate prepared in 20 mM Tris, containing 10% glycerol, 150 mM NaCl, and 1% Triton X-100, pH 7.8 at 4 °C. After centrifugation (1000 × *g*), 200 μl of the supernatant was added to 1750 μl of reaction buffer (125 mM Tris, pH 9.0, and 1 mM EDTA) and 17 μM FAAH substrate decanoyl *m*-nitroaniline (*m*-NA) (Cayman Chemical Company, Ann Arbor, MI, USA) and absorbance was measured at 410 nm [37]. Results were normalised to protein content.

2.5.4. Determination of endocannabinoid receptors expression

Western blot analysis of cell protein was performed according to Eissa and Seada [26]. Membrane fractions were mixed with Laemmle buffer containing 5% 2-mercaptoethanol, heated at 95 °C for 10 min, and separated by 10% Tris-Glycine SDS-PAGE. Separated proteins were electrophoretically transferred into nitrocellulose membrane. The blotted membrane was blocked with 5% skim milk in TBS-T buffer (5% Tween 20) for 1 h. Primary antibodies were raised against CB1, CB2 (Santa Cruse Biotechnology, Santa Cruz, CA, USA), VR1 (Abcam Inc., Cambridge, MA, USA) and GPR55 (Sigma-Aldrich, St. Louis, MO, USA) were used at a concentration of 1:1000. Protein bands were visualised using the BCIP/NBT Liquid substrate system (Sigma-Aldrich, St. Louis, MO, USA) and were quantitated using the Versa Doc System and Quantity One software (Bio-Rad Laboratories Inc., CA).

3. Statistical analysis

Statistical analysis was performed using StataIC 13.0. Data obtained in the current study are expressed as mean and ± SD. Because the tissue samples were paired (tumour-free and tumour tissues from the same individual) the comparison of tumour-free and tumour tissues between different tumour subtypes was carried out using an ANOVA. The subgroup analyses of the paired samples in terms of antioxidant expressions were done by paired *t* test. P value < 0.05 was considered statistically significant.

4. Results

4.1. Antioxidant capacity

Obtained results demonstrate varying degrees of redox disorders depending on the lung cancer subtype. In the case of GSH-Px activity, a 4- and 5-fold decrease compared with control tissue was observed in SCC and AC, respectively. A similar dependence was also recorded for CAT activity, where a 30% and 60% decrease was observed in SCC and AC, respectively (Table 1). On the other hand, GSH level was diminished to approximately 60% in both subtypes of lung cancer (Table 1).

Table 1
The activity/level of antioxidant enzymes (GSH-Px and CAT) and nonenzymatic antioxidant (GSH) in the lung cancer tissue [SCC, n=38 and AC, n=34] and lung tissue.

Analysed parameter	SCC n=38		AC n=34	
	Lung tissue	Cancer tissue	Lung tissue	Cancer tissue
GSH-Px mU/mg protein	81.1 ± 3.8	21.2 ± 1.2[a]	69.3 ± 5.1	12.4 ± 0.8[a,b]
CAT U/mg protein	2.15 ± 0.13	1.45 ± 0.07[a]	1.81 ± 0.11	0.71 ± 0.03[a,b]
GSH [nmoles/g tissue]	1.37 ± 0.10	0.82 ± 0.07[a]	1.29 ± 0.07	0.80 ± 0.04[a]
GSSG [nmol/g tissues]	0.038 ± 0.002	0.063 ± 0.007[a]	0.044 ± 0.006	0.053 ± 0.009[b]
GSH/GSSG	36.05 ± 0.12	13.0 ± 0.3[a]	29.3 ± 1.4	15.4 ± 1.9[a]

[a] Statistically significant differences vs. control group, $p < 0.05$.
[b] Statistically significant differences vs. SCC group, $p < 0.05$.

4.2. Nrf2/ARE pathway

The antioxidant defence of non-small cell lung cancers is also dependent on the activity of the Nrf2/ARE pathway (Fig. 1). SCC and AC were both characterised by a 2-fold increase in the expression levels of the transcription factors Nrf1 and Nrf2, and their target HO-1. Furthermore, a 3- and 6-fold decrease in the level of the Nrf2 inhibitor Bach1 was observed in SCC and AC, respectively, compared with control tissues. An increase in the expression levels of proteins that activate Nrf2 - p21 and p62 was observed: the expression of p21 increased by ~25% in SCC and 90% in AC, while the expression of p62 was enhanced by ~50% and 100% in SCC and AC, respectively. Moreover, a 2-fold increase in the level of phosphorylated ERK1/2 was observed in AC compared with SCC.

4.3. DNA modifications

Changes in the antioxidant defence of NSCLC were accompanied by an increase in the levels of DNA damage markers, such as 8-OHdG, which increased by 2.3- and 4-fold in SCC and AC, respectively (Table 2). The level of DNA methylation in the tested cancer tissues was also higher by 1.4- and 2.4-fold in SCC and AC, respectively, compared with control tissues (Table 2).

4.4. PUFAs and lipid peroxidation products

Development of NSCLC leads to disturbances in the membrane phospholipid structure observed as changes in the levels of phospholipid fatty acids and lipid peroxidation products (Table 3). Our results show the stronger decrease in docosahexaenoic acid levels in AC (up to 50%) than in SCC (to 60%), while the decrease in both arachidonic and linoleic acid levels was at the same level (approximately 60%) in both cancer subtypes. In both types of cancer an increase in the levels of 4-HNE and MDA was observed.

Table 2
The level of DNA modifications [8-OHdG and N^7-MeG] in the lung cancer tissue [SCC, n=38 and AC, n=34] and lung tissue.

Analysed parameter	SCC n=38		AC n=34	
	Lung tissue	Cancer tissue	Lung tissue	Cancer tissue
8-OHdG/10^5dG	1.99 ± 0.70	4.59 ± 0.62[a]	2.37 ± 0.82	9.41 ± 2.38[a,b]
N^7-MetG (μmoles/mol G)	15.01 ± 2.22	20.91 ± 1.06[a]	13.54 ± 1.66	32.74 ± 2.81[a,b]

[a] Statistically significant differences vs. control group, $p < 0.05$.
[b] Statistically significant differences vs. SCC group, $p < 0.05$.

Table 3
The level of phospholipid fatty acids [ph-LA, ph-AA, and ph-DHA], lipid peroxidation products [4-HNE, MDA, 4-ONE], and 4-HNE – protein adducts in the lung cancer tissue [SCC, n=38 and AC, n=34] and lung tissue.

Analysed parameter	SCC n=38		AC n=34	
	Lung tissue	Cancer tissue	Lung tissue	Cancer tissue
ph-LA [μg/ml]	20.95 ± 2.27	11.98 ± 1.70[a]	18.92 ± 2.55	11.36 ± 5.97[a]
ph-AA [μg/ml]	46.17 ± 3.51	27.30 ± 4.98[a]	44.10 ± 5.01	33.81 ± 4.29[a]
ph-DHA [μg/ml]	11.72 ± 1.70	7.06 ± 1.02[a]	10.04 ± 1.13	4.99 ± 0.97[a,b]
4-HNE [nmoles/ml]	3.46 ± 1.31	9.63 ± 2.60[a]	3.42 ± 1.65	8.08 ± 2.87[a]
MDA [nmoles/ml]	17.24 ± 2.53	70.10 ± 9.73[a]	19.71 ± 3.24	115.52 ± 13.65[a,b]
4-ONE [nmoles/ml]	0.23 ± 0.04	0.52 ± 0.30	0.30 ± 0.08	0.59 ± 0.56
4-HNE – protein adducts [pmoles/mg protein]	50.07 ± 9.81	73.02 ± 8.31[a]	59.87 ± 3.65	103.49 ± 8.79[a,b]

[a] Statistically significant differences vs. control group, $p < 0.05$.
[b] Statistically significant differences vs. SCC group, $p < 0.05$.

The level of MDA was nearly 4-fold higher in SCC than in control tissues, while in AC the levels were 6-fold higher compared with control tissues; similarly, the levels of 4-HNE were 2.7- and 2.3-fold higher in SCC and AC, respectively (Table 3). The increase in the level of 4-HNE-protein adducts was higher in AC than in SCC (increase of approximately 46% and 83% for SCC and AC, respectively) (Table 3). Immunohistochemical analysis revealed that both SCC and AC tissues had abundant 4-HNE-protein adducts present in necrotic tissues, while strong difference was noticed between stromal and cancer cells (Fig. 2): stromal cells were mostly positive for the 4-HNE-protein adducts, while only some of malignant cells exhibited prominent presence of the 4-HNE-protein adducts.

4.5. Endocannabinoid system

Cancer development leads to changes in the endocannabinoid system. The strong difference between the tested cancer subtypes

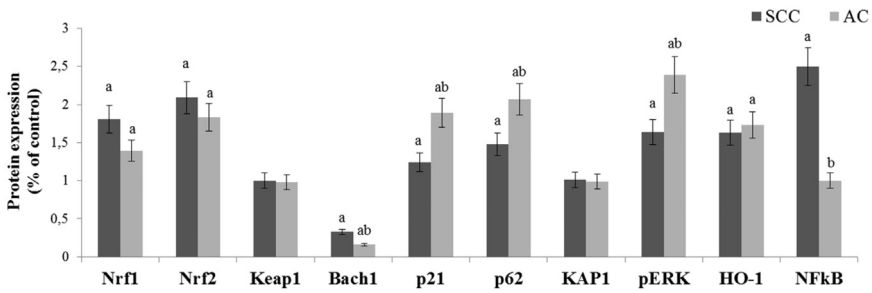

Fig. 1. The expression of the transcription factors [Nrf1 and Nrf2], their activators and inhibitors, and the inflammation factor NFκB as the ratio of lung cancer tissues [SCC, n=38 and AC, n=34] and lung tissues. a – statistically significant differences vs. control group, $p < 0.05$; b – statistically significant differences vs. SCC group, $p < 0.05$.

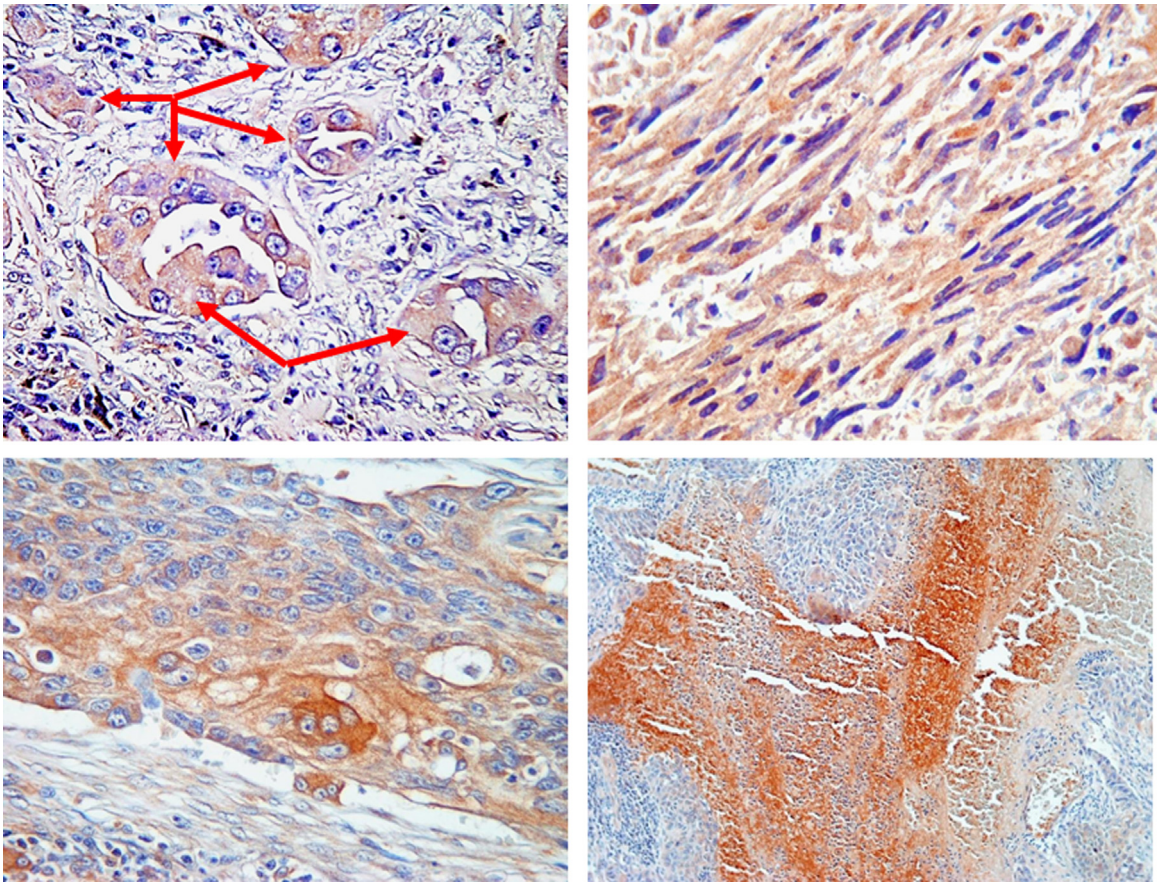

Fig. 2. Immunohistochemical detection of the 4-HNE-protein adducts in SCC and AC tissues. Immunohistochemistry for 4-HNE-His presence detection by specific monoclonal antibodies was visualised by brown reaction of DAB (3,3′-diaminobenzidine), with the negative counterstaining with hematoxylin giving blue coloured reaction. In malignant AC cells (upper left picture, 400 ×) and SCC cells (lower left picture, 400 ×) abundant presence of 4-HNE-modified proteins was observed in some cells (brown colour, indicated by arrow), while the other cells appeared to be free of the proteins modified by the aldehyde (blue colour). Stromal cells were mostly positive for the 4-HNE-protein adducts (upper right picture, 400 ×), although the nuclei of these cells did not show any presence of the 4-HNE-His adducts, as was also observed for malignant cells. The most abundant 4-HNE-His adducts were found in necrotic regions of the cancer (lower right picture, 100 ×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

is evident in the case of the endocannabinoids (AEA and 2-AG) level, where 1.8- and 2.3-fold increases in SCC and 1.44- and 2.3-fold decreases in AC samples were observed (for AEA and 2-AG, respectively) (Fig. 3). At the same time, a significant increase in the activity of the enzymes responsible for the degradation of endocannabinoids in both cancer subtypes was observed (Fig. 3). Approximately 30% and 22% increases in FAAH activity and >7- and 1.5-fold increases in MAGL activity were found in SCC and AC, respectively. Moreover, the decrease in the levels of endocannabinoids in AC was also accompanied by an increase in the expression of their receptors CB1/2 (approximately 50% for CB1 and 20% for CB2), which was not found in SCC. However, in both SCC and AC, a significant increase in the expression of other endocannabinoid receptors – TRV1 (33% for SCC and 56% for AC) and GPR55 (47% for SCC and 92% for AC) was observed (Fig. 4).

4.6. Inflammation process

The expression level of the inflammation factor NFκB also strongly shows the difference between the tested cancer subtypes (Fig. 1). A 4-fold increase in the level of NFκB was found in SCC compared with the controls, while no significant changes were found in AC.

5. Discussion

Lung tissue is continuously in contact with higher oxygen pressure than other tissues and, due to its large surface area, exhibits particular susceptibility to ROS [38]. Moreover, the lung is a major target of air pollutants, several of which are, or are metabolised to, electrophiles leading to malignant transformation [2]. Therefore, lung cancer tissue is characterised by elevated oxidative stress [39], particularly in situations when antioxidant defence mechanisms are inefficient. Although some studies have shown increased antioxidant activities in blood cells from patients with lung cancer [40], others have reported reduced antioxidant activities in lung tumour tissues [41,42]. Impairment of the antioxidant defence leads to the damaging effects of electrophiles that seek areas of high electron density, such as the purine and pyrimidine bases of DNA resulting in 8-OHdG generation as well as direct modifications of DNA by carcinogens with elevated levels of methylated guanine as shown in this paper. Our results indicate that the extent of modifications was higher in AC, which represents the more aggressive form of lung cancer.

In human lung cancer cells, the higher level of DNA modifications were associated with lower GSH-Px activity and reduced gene expression of GSH-Px in normal bronchial epithelial cells, which has been implicated as a risk factor for the development of lung cancer in cigarette-smokers [43,44]. Our results indicate that in the development of lung AC, more than in lung SCC, the levels of both peroxides, including lipid peroxide degradation enzymes GSH-Px and CAT, are diminished. Moreover, the levels of reduced glutathione, a co-substrate of GSH-Px, are also decreased in lung cancer tissue, which promotes a decrease in the degradation of

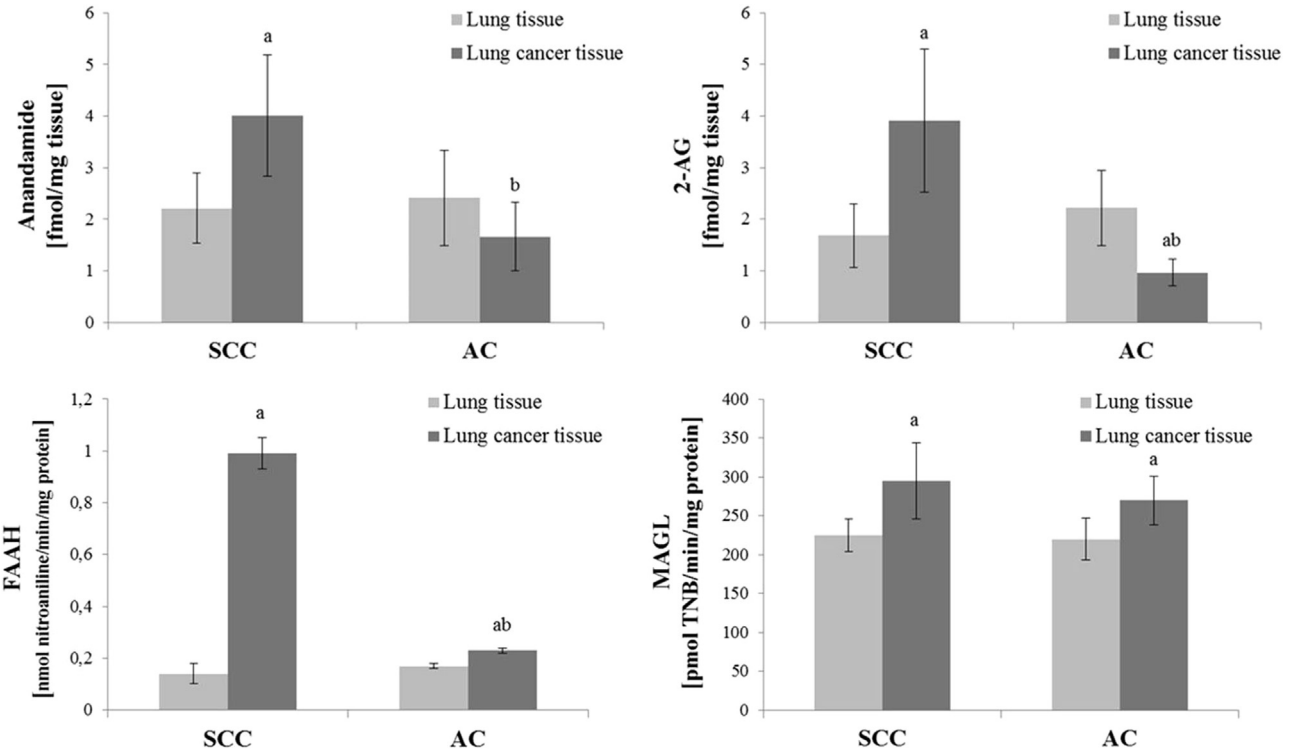

Fig. 3. The level of the endocannabinoids [AEA and 2-AG] and the activity of the enzymes responsible for endocannabinoids degradation [FAAH and MAGL] in the lung cancer tissue [SCC, n=38 and AC, n=34] and lung tissue. a – statistically significant differences vs. control group, $p < 0.05$; b – statistically significant differences vs. SCC group, $p < 0.05$.

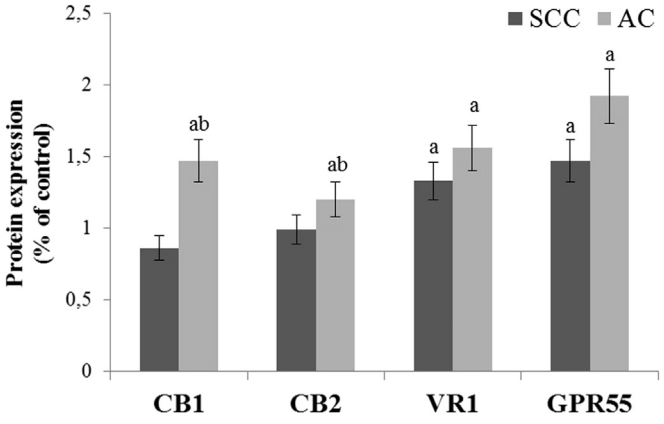

Fig. 4. The expression of the endocannabinoids receptors [CB1, CB2, VR1, and GPR55] as the ratio of lung cancer tissues [SCC, n=38 and AC, n=34] and lung tissues. a – statistically significant differences vs. control group, $p < 0.05$; b – statistically significant differences vs. SCC group, $p < 0.05$.

peroxides in malignant carcinogenesis. The consequence is an enhanced level of hydrogen peroxide in human lung squamous cell carcinoma [45], as well as modifications of biomembrane phospholipids in AC and SCC tissues leading to enhanced electrophilic lipid peroxidation products such as MDA, 4-HNE and 4-ONE, as observed in our study.

Cellular antioxidant defence mechanism is also connected with the transcription factor Nrf2 as well as its activators and inhibitors [46]. Nrf2 activates cellular rescue pathways against oxidative injury, inflammation, apoptosis, and carcinogenesis through transcriptional induction of a broad spectrum of genes involved in xenobiotic detoxification and antioxidant protection [4]. Moreover, it has been demonstrated that Nrf2 promotes the survival not only of normal cells but also cancer cells [47]. Therefore, prolonged activation of Nrf2 favours the progression of several types of cancer, including lung cancer [48] and the prognosis of such patients is poor, partly due to the ability of Nrf2 to enhance cell proliferation, chemoresistance and radioresistance [49,50]. Elevated levels of Nrf2 promote generation of antioxidants such as GSH, and maintain the redox balance as well as the transcription of several genes involved in the proliferation of cancer cells [51]. It was revealed that glutathione is a critical antioxidant participating in cell proliferation and that its level is elevated in A549 lung cancer cells [51]. However, our results have shown the diminished levels of GSH in both subtypes of cancer - AC and SCC, which indicates the lower proliferation in lung cancer tissues. Activity of Nrf2 is primarily regulated by Keap1, which anchors it within the cytoplasm, targeting it for ubiquitination and proteasomal degradation, thus repressing its ability to induce cytoprotective genes [5]. The degradable complex consists of Nrf2, Keap1 and Cul3, and somatic mutations in *Keap1* and *Nrf2* genes, which often lead to disturbances in the formation of these complexes, were shown in different cancers including lung SCC [51,52]. Moreover, epigenetic modifications in Keap1 have also been shown to promote the accumulation of free Nrf2 in NSCLC tissue [53].

An examination of NSCLC cell lines showed that different subtypes of lung cancer are characterised by varying degrees of Nrf2 activation and higher activation is associated with higher resistance to anticancer drugs as in the case of A549 cells [54]. This study shows that this dependence occurs not only in the case of cell lines but also in human tissues, where Nrf2 activation is stronger in SCC than in AC. Moreover, no significant changes in Keap1 expression were observed in either of the examined cancer subtypes. Therefore, high Nrf2 activity can be explained by *Keap1* gene mutations leading to non-conservative amino acid substitutions and nonsense mutations in 50% of the sequenced NSCLC cell lines [55]. In the present study, we also show that p21 – a cytosolic Nrf2 activator, is expressed at higher levels in AC. Transcription of the *p21* gene is induced by DNA damage as a consequence of the accumulation of p53, and mediates p53-induced growth arrest to

avoid the replication and subsequent propagation of potentially hazardous mutations [56]. Other studies have shown that p21 is also induced through a p53-independent mechanism in response to oxidative stress [57]. Furthermore, the accumulation of p53 is strongly connected with DNA modifications and even mutations [58]. Moreover, it was indicated that in different subtypes of cancers including lung cancers, the promoter region of the Keap1 gene is hypermethylated [53], which is in agreement with our results showing enhanced levels of MetG in lung cancer tissues. Moreover, these findings show that the expression level of another Nrf2 activator, p62, is also enhanced in lung AC tissues and to a lower extent in SCC tissues. It was revealed earlier that this activator may directly interact with Keap1 and sequestrate Keap1 into phagosomes [59]. In the mouse model of Ras-induced lung adenocarcinoma, the overexpression of p62 resulted in the activation of NFκB [60]. In this study, enhanced levels of NFκB were observed only in SCC tumours.

Keap1, as a cysteine-rich protein, is highly susceptible to interactions with electrophilic compounds including lipid peroxidation products such as reactive aldehydes [61]. The levels of 4-HNE, 4-ONE and MDA are significantly enhanced in lung cancer tissues. Following cysteine modifications, conformational changes within the Keap1 homodimer cause the dissociation of Nrf2 and its translocation to the nucleus and its transcriptional activity, leading to enhanced expression of OH-1, as observed in this paper. It was shown that the release of Nrf2 from the complex with Keap1 ensures a higher survival rate in cancer cells, especially under stress conditions [62]. In particular, 4-HNE interferes with the expression of many proteins connected with this transcription factor, especially the Nrf2 inhibitor Bach1 [63].

The observed increase in 4-HNE is associated with a decreased level of AA and LA, which are the major sources of 4-HNE generated during lipid peroxidation, suggesting the metabolic preference toward lipid peroxidation of these PUFAs as has been observed before in chronic obstructive pulmonary disease [64]. However, such a correlation is observed in this study for the first time in lung cancer tissue samples. Some of the above aldehydes may diffuse to longer distances than ROS and act as secondary messengers of free radicals that can propagate oxidative damage, in particular if bound to proteins, which is the usual feature of HNE. It was implied that aldehydes as lipid peroxidation products only elicit damage, yet their impact could be more varied and depend on concentration and the protein targets involved [65]. The reactive aldehydes possess an aldehydic functional group and/or an α,β- unsaturated bond which allows them to react by both Schiff's base formation and Michael addition, respectively. Michael addition occurs by reaction of the electrophile with the nucleophilic cysteine, lysine and histidine residues of protein to form stable covalent adducts [66]. Exposure of biological systems to these electrophiles can modify a subset of proteins, generating a variety of intra- and intermolecular covalent adducts relevant for major cellular metabolic and growth regulating processes.

Therefore, it should be stressed that the increase in the 4-HNE-His protein adducts in lung cancer tissues that is observed for the first time in this study is, in particular, associated with the majority of stromal cells and with some cancer cells. Similar differential expression of proteins and reactive aldehydes was previously noticed in exposure to acrolein in human prostate carcinoma [67], where it seems to be important for the spread of cancer and its recurrence. On the other hand, production of 4-HNE-His adducts by non-malignant cells of the cancer tissue could be a relevant mechanism of tumour growth control, not only because it is well known that HNE can act as a growth regulating factor of cancer and of non-malignant cells in specific relation to their antioxidant capacities [68], but also because it was recently found that it can specifically suppress membrane-associated catalase in the cancer cells, thus inducing both apoptosis and necrosis of malignant cells [69,70]. Our findings of the abundance of 4-HNE-His adducts in necrotic lung cancer specimens favour these options.

Moreover, aldehydic-protein adducts, denoted also as advanced lipoxidation-end products (ALE), are potential targets for the cellular and humoral immune response. Bound to proteins, these aldehydes induce immune responses by breaking the tolerance for cellular proteins. It has been shown that the modification of physiological proteins by lipid peroxidation products indeed results in the autoimmune reaction to their ALEs [71]. Although the production of autoantibodies against cancer antigens is not considered to be a very effective defence against cancer, the cellular reaction mediated by macrophages, lymphocytes, and granulocytes could be an important defence mechanism, which will be associated with the production of reactive aldehydes [72,73]. In addition to histidine and lysine, another target for reactive aldehydes are cysteine residues in proteins but also in GSH - an abundant low molecular mass thiol free radical scavenger [74] and that the GSH level was found to decrease in parallel with increased level of aldehydes in lung cancers. The pathophysiological consequences of such chemical reactivity of these electrophiles are prominent [75] because it is well recognised that the thiol groups often act as redox switches, controlling cell signalling and metabolism [76]. It should be noted that the above mentioned aldehydes may induce synovial intrinsic inflammation by activating NFκB pathways what may lead to cell apoptosis [77] and that the expression of NFκB was enhanced in lung cancer tissues in this study. Because NFκB transcribes various structural proteins, growth factors, pro-inflammatory cytokines and apoptotic proteins, it is certain that lipid peroxidation products are involved in the development of cancer. Moreover, the interaction with protein kinases such as SRC and PLC could change cellular calcium signalling leading to the activation of the caspases involved in cell death [78]. Therefore, both relatively moderate aldehydic modifications of some major proteins as well as abundant accumulation of non-specific aldehyde-protein adducts may be an underlying factor in the development of many disorders, in particular cancer [79]. This conclusion can also be supported by the enhanced levels of other lipid peroxidation mediators, such as MDA, of which increased levels have so far only been examined in the plasma of NSCLC patients [80].

ROS/RNS and inflammatory mediators are implicated in the functioning of the endocannabinoid system by changing the generation and degradation of AEA and 2-AG as well as the activation of cannabinoid receptors (CB1, CB2, TRPV1, GPR55) [81]. It was shown that the endocannabinoids exert potential anti-tumour effects in various cancer cells [82], but the endocannabinoid system may be dysregulated in a number of cancers [83]. Endocannabinoids modulate ERK and ROS pathways leading to apoptosis in normal and cancer cells [84]. The results of this study showed that the altered levels of anandamide and 2-arachidonoylglycerol are dependent on the subtype of lung cancer, with increase in SCC and decrease in AC tissues being observed. Previous studies showed that treatment of NSCLC cell lines with AEA or AEA degradation inhibitor [FAAH inhibitor] significantly decreased the growth of tumour cells [85] and increased FAAH expression was indicated only in prostate adenocarcinoma [86]. Therefore, the increase observed in this study in the levels of endocannabinoids in SCC indicates that the growth of this cancer should be diminished. Endocannabinoids are ligands of cannabinoid receptors, CB1, CB2, TRV1 and GPR55, among which expression of CB1/2 is decreased in SCC. This indicates that anandamide activity is involved in independent mechanisms, e.g., via COX-2 metabolism or by TRV1 or GPR55 receptor activation [84]. However, the lung AC was characterised by higher expression of all

examined endocannabinoids receptors. It was reported that lower levels of endocannabinoids along with higher expression of receptors are associated with the increased viability of cancer cells [87]. Such a situation was observed only in AC, which is known to be more aggressive than SCC. An increase in the levels of endocannabinoids in SCC leads to death. Data from the literature confirm that endocannabinoids control the fundamental processes of cell homoeostasis and neoplastic transformation [86], which agree with our results. Moreover, it was shown that the majority of NSCLCs overexpress EGFR, which has been correlated with a poor prognosis and resistance to chemotherapy and that CB1 or CB2 activation attenuated EGF-induced morphological changes, such as cell elongation and generation of protrusions leading to the rounding and reduced motility of NSCLC cells [88]. Furthermore, signalling studies indicated that CB1 and CB2 may also act by phosphorylation of ERK1/2, JNK1/2 and AKT, and lead to Nrf2 activation. Moreover, endocannabinoid receptors may differentially regulate the generation of ROS. It was shown that activation of CB1 enhances oxidative stress, whereas CB2 and TRPV1 activation leads to suppression of oxidative stress [88]. Therefore, small molecular weight synthetic cannabinoids inhibit growth, migration and invasion of NSCLC cells in vitro as well as in vivo in a mouse model [89].

Finally, we conclude that lipid metabolism is not altered in the same way in SCC and AC. Namely, lipid peroxidation appears to be more involved in AC development, while endocannabinoids participate more in SCC growth and development. Therefore, understanding the relation between the various lipid mediators and antioxidants in different lung cancer subtypes may give the opportunities for further research on the targeted anticancer therapy.

Conflict of interest

The authors have no conflicts of interest to declare.

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