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PLEIOTROPIC EFFECTS OF CALCIUM D-GLUCARATE ON CHEMICALLY-INDUCED LUNG CARCINOGENESIS IN A/J MICE

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ABSTRACT

Calcium D-glucarate (CG) reportedly suppresses benzo[*a*]pyrene (B[*a*]P)-induced lung carcinogenesis in A/J mice, by inhibiting the enzyme ß-glucuronidase (ß-G). The protective mechanism conferred by ß-G inhibition operates, in part, through a reduction in oncogene mutations and suppression of cell proliferation and chronic inflammation. The aim of the current study was to provide evidence on the ability of CG not only to suppress cell proliferation and inflammation but also to induce apoptosis during post-initiation stages of B[*a*]P-induced lung tumorigenesis in A/J mice. Two doses of 3 mg of B[*a*]P were given intragastrically to A/J mice two weeks apart. CG administration in the AIN-93G diet (2.0% and 4.0%, w/w) started at 2 weeks after the second dose of B[*a*]P. There was no change in the calcium content of CG diets in comparison with the control AIN-93G diet. Mice were scarified at week 20 and estimation on serum ß-G level and proliferation, inflammation, and also chosen apoptosis marker analyses were performed. The proapoptotic effects of CG were investigated immunohistochemically using antibodies against active fragments of caspase 9 and poly(ADP-ribose) polymerase-1 (PARP-1), respectively. Both 2% and 4% diets reduced significantly not only the number of lung adenomas and hyperplasia 4 months after B[*a*]P gavaging. These diets also increased, in a dose related fashion, levels of active fragments of caspase 9 as well as PARP-1 in the lung tissue. The activity of above apoptosis-related proteins increased up to over 99% and 152%, respectively in the lung tissues of A/J mice treated with B[*a*]P and dietary D-glucarate compared to mice treated with B[*a*]P. At the same time, approximately 47% inhibition of cyclooxygenase-2 (COX-2) was estimated in the epithelial cells lining the bronchioli and bronchus as well as type 2 alveolar cells in the group of mice on the 4% CG diet. The BrdU-labeling indices for the alveolar/bronchiolar hyperplasia and adenoma cells were also determined. Post-initiation dietary CG caused significant and dose related decrease in the number of proliferating pneumocyte type 2 cells (*i.e.*, up to 37%) and macrophages (*i.e.*, up to 44%) compared to those in alveoli of the B[*a*]P-treated A/J mice. We conclude that post-initiation D-glucarate may inhibit B[*a*]P-induced carcinogenesis in A/J mice not only by suppressing cell proliferation and inflammation but also by programmed cell death induction.

KEYWORDS: Mouse lung carcinogenesis, calcium glucarate, ß-glucuronidase, proliferation, inflammation, apoptosis.

INTRODUCTION

Lung cancer is the leading cause of cancer- related death in both men and women in the western world.[1] The high mortality associated with this cancer is mainly due to the fact that vast majority of this disease cases are detected usually in an advanced stage. Based on histopathological criteria lung cancer is divided into non-small cell lung cancer (NSCLC; ~80%) grouped into subtypes: adenocarcinomas (~50%), squamous cell carcinoma (~30%), large cell lung carcinoma and small cell lung carcinoma (SCLS; ~20%).[2] This distinction is important clinically to their different responses to therapy. Cigarette smoking is the principal cause of this cancer; it is estimated to be responsible for 85% of lung cancer types.[3] Among the multiple components of tobacco smoke a large number are likely to be involved in lung cancer inductions.[4] Polycyclic aromatic hydrocarbons and nitrosamines are likely to play important roles. Due to appalling statistics (a relatively low 5-year survival rate) it is necessary to improve diagnostics and develop not only novel and effective lung cancer therapeutics[5] but also to introduce new chemopreventive strategies.

It is generally accepted that fruits and vegetables intake may decrease the risk of several human cancers. One of hypotheses suggests that inhibition of acid hydrolase – ß-glucuronidase (ß-G) is involved natural food components. ß-G is lysosomal enzyme found in most

organs such as the liver, spleen, kidney, intestine, endocrine and reproductive ones.[6] This enzyme hydrolyzes glucuronic acid from substrate complexes with carcinogens, steroid hormones, promoting agents.[7] Released constituents, after deglucuronidation, gain ability to recirculate and interact with cells. Among numerous natural food substances tested in experimental studies, D-glucaric acid and its derivatives indicate ß-G inhibitor activity in humans and are suggested to reduce cancer risk.[6-8]

Labilization of lysosomal enzymes is associated with the general process of inflammation. Also, the release of hydrolytic enzymes from the lysosomal compartment to the cytosol is an important initiating event in the apoptotic process. Serum levels of proinflammatory cytokines such as interleukin-1 and C-reactive protein correlate well with ß-G activity in the serum from patients with inflammatory disorders.[9] Thus, ß-G is a potential biomarker useful in monitoring pulmonary inflammation caused by human exposure to different environmental carcinogens including the tobacco smoke.[10]

D-glucaric acid is converted into D-glucaro-1,4-lactone, the substance which competitively inhibits ß-G and reveals the capacity to reduce mammary, skin, liver, colon, lung and oral cancers in animals.[11-18] Interestingly, these natural, nontoxic agents have been also used with several therapeutic approaches including cholesterol reduction,[19,20] diabetes treatment[21], rheumatoid arthritis[22] and protective activity against oxidative/nitrative damage of plasma protein.[23]

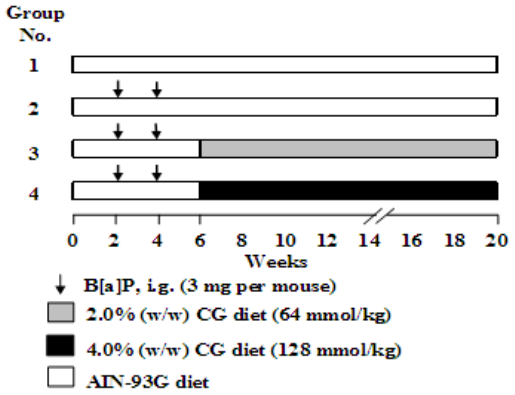
Our previous study revealed that calcium D-glucarate (CG) inhibited benzo[*a*]pyrene (B[*a*]P) A/J mouse lung carcinogenesis.[17] To farther characterization the role of CG in (B[*a*]P)-induced mouse lung carcinogenesis, here we demonstrated by new panel of biological markers that post-initiation dietary with the above agent may inhibit carcinogenesis not only by the decreasing cell proliferation and inflammation but also by apoptosis induction.

MATERIALS AND METHODS

ANIMALS, CARCINOGENIC AND CHEMOPREVENTION PROTOCOLS

Female A/J mice of 4 weeks of age were obtained from Jackson Laboratory (Bar Harbor, MA). Mice were fed the AIN-93G purified diet (Dyets, Bethlehem, PA) and maintained under the following standard conditions: 20±2°C, 50±10% relative humidity and 12-h light, 12-h dark cycle.

Two doses of 3 mg of benzo[*a*]pyrene (B[*a*]P) in 0.25 ml of cottonseed oil or cottonseed oil only (vehicle) were given intragastrically to 6-week-old mice. The time interval between the first and second doses of B[*a*]P was 2 weeks. Calcium D-glucarate administration in the AIN-93G diet started at 2 weeks after the second dose of B(*a*)P (Scheme 1.). There was no change in the calcium content of the CG diets in comparison with the control AIN-93G diet. The animals were sacrificed at 16 weeks after the second dose of B[*a*]P. Immediately upon sacrifice, blood was collected by cardiac puncture and lungs were perfused with cold phosphate buffered saline and harvested. Normal lungs from vehicle-treated mice and the lungs from carcinogen-treated mice were frozen in liquid nitrogen and stored at -80°C.



Scheme 1: Details of experimental design.

ß-GLUCURONIDASE ASSAY

β-glucuronidase (ß-G) activity from serum was determined using the following method modified from the Sigma β-glucuronidase kit (Sigma-Aldrich Company, St. Louis, MO), because the kit had been discontinued. Fifty µl of serum was used in the assay with a corresponding reduction in the volumes of the enzyme substrate, the acetate buffer and water. The volume of the AMP buffer (stop reagent) was reduced by 20%. Serum was incubated with phenolphthalein glucuronic acid (Sigma), the ß-G substrate, at pH 4.5 for 4 h at 37°C. At exactly 4 h, the reaction was stopped using an alkaline buffer. Under standard conditions, ß-G cleaves phenolphthalein glucuronic acid liberating free phenolphthalein.

A DU640 Spectrophotometer (Beckman Instruments, Fullerton CA) was used to monitor the intensity of the resulting pink color which is proportional to β-G activity. We determined enzyme activity, expressed as µg phenolpthalein released /ml of serum/h at 37°C, from standard curves.

BrdU POSITIVE CELL NUMBER

Bromodeoxyuridine (BrdU) was administered intraperitoneally to 5 mice from each experimental group, 2 h before sacrifice at a dose of 50 µg/g of body weight. To study pulmonary hyperproliferation induced by B[*a*]P, the BrdU-labeling indices for the alveolar/bronchiolar hyperplasia and adenoma cells were determined immunohistochemically. The 5-µm paraffin sections of each lung were immunostained with the use of NCL-BrdU mouse IgG1 antibody (Novocastra Laboratories, UK) and the avidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA).

The proliferation index, *i.e.*, the percentage of BrdU-positive alveolar or bronchiolar epithelial cells, was determined using an Olympus BX45 microscope. For each slide, at least 10 bronchioli and 10 regions of alveoli were counted; the total number of counted cells was 2000. The number of BrdU-positive cells was performed as described previously.[24]

PNEUMOCYTE TYPE 2 AND MACROPHAGE NUMBER

Of the several cell types in the alveolar wall, the type 2 pneumocyte has been shown to be a target cell for benzo[*a*]pyrene in the mouse lung.[25] Strain A/J is highly susceptible, with a tumor multiplicity of 12-24 tumors per mouse 14-16 weeks after the second of two 3 mg *per oral* doses of B[*a*]P administered two weeks apart. The alveolar wall cells examined included type 2 pneumocytes and macrophages. Relative proportions of BrdU-labeled cell types were determined by classifying 500 labeled cells from each control or experimental group (100 cells/animal) into one of the following categories: macrophages (large irregular nucleus and non-vacuolated cytoplasm) and type 2 pneumocytes (rounded nucleus and vacuolated cytoplasm). Differential counts were done on control groups and on experimental groups at sacrifice. Mean values were compared among groups using Student's *t*-test with $P<0.05$ as the minimum acceptable level for the establishment of statistically significant differences.

IMMUNOHISTOCHEMISTRY ASSAYS

Murine lung sections were deparaffinized, rehydrated, and endogenous peroxidase activity was inhibited with 3% H_2O_2, followed by antigen retrival. Slides were then blocked with 2.5% normal goat serum (Vector Laboratories, Burlingame, CA). For the immunocytochemical localization of β-G, COX-2, cleaved caspase-9, cleaved PARP-1 in paraffin sections, the avidin-biotin complex technique (Vector Laboratories) and 3,3-diaminobenzidine as the peroxidase substrate (Sigma) was employed according to the manufacturer's procedure. The antibodies recognizing the cleaved caspase-9 (37kDa), cleaved PARP-1 (89kDa) were purchased from Cell Signaling, Danvers, MA, and anti-COX-2 from Cayman Chemical Co., Ann Arbor, MI. At least 20 different sections on one slide were viewed, counted, and photographed on an Olympus BX45 microscope.

STATISTICAL ANALYSIS

Statistical analysis was done with the use of ANOVA: two-factor without replication and Student's *t*-test. Values are means ±SD. Both CG groups were compared to the B[*a*]P group.

RESULTS AND DISCUSSION

The chemically-induced mouse lung cancer model provides an important system for studying mechanisms involved in the different stage of carcinogenesis and for assaying various carcinogenic agent properties, as well as for searching compounds that may inhibit cancer formation and malignant conversion. There is now a growing evidence for the possible control of various stages of the cancer induction by inhibition of β-glucuronidase[6,14] with D-glucaric acid (GA) derivatives, mainly with its salts such as calcium D-glucarate. These agents besides being found in fruits and vegetables being endogenously synthesized in mammals, are available in supplements and beverages.[10,20] A large number of *in vitro* and *in vivo* reports have been revealed the potential of GA derivatives as chemotherapeutic and chemopreventive compounds with no side-effects.[11,13,16,18,,20,26,27]

It seems that chemopreventive and anticancer mechanisms of GA derivatives are mainly associated with hormonal modifications of body and proliferative status of target organs, their capacity for carcinogen and neoplasia promoter neutralization, limitation of oncogenic mutations, as well as ability for inflammation diminishing and induction of cancerous cell apoptosis.[7,8,10,12,17] Studies from this laboratory have been described that dietary with GA derivatives is an effective preventive agents against cancer development in some rodent organs including mammary glands, colon, skin, lung.[12,15,17,28]

Activity of calcium glucarate is believed to drive from gradual conversion of about one-third of this agent to the β-glucuronidase inhibitor, D-glucaro-1,4-lactone, at the low pH of the stomach. D-glucaro-1,4-lactone, competitively inhibits β-G and has been indicated to reduce chemically induced mammary, liver, skin, oral carcinogenesis in animals.[12,14,18,26]

Effect of calcium glucarate on β-glucuronidase activity and cell proliferation in B[*a*]P-treated mice

The results of the current investigation strongly suggest that increasing concentration of CG (2% and 4%) in AIN-93 diet in B[*a*]P-treated female A/J mice influenced on gradual decrease of β-G activity in their serum, i.e. about by 24% compared to B[*a*]P group (Table 1).

Table 1: Inhibitory effect of dietary calcium D-glucarate (CG) on serum β-glucuronidase activity in B[*a*]P-treated female A/J mice. β-Glucuronidase values are means ±SD. *β-Glucuronidase at sacrifice. MSU=Modified Sigma Units. NS=not significant. Statistical analysis was done with the use of ANOVA: two-factor without replication. The B[a]P group was compared to the control group; the CG groups were compared to the B[*a*]P group (% of inhibition).

MICE GROUP (N)	CARCINOGEN	DIET	SERUM b-GLUCURONIDASE * (MSU/ML)	INHIBITION %	P-VALUE
Control (N=30)	None	AIN-93G	22 ±3	-	-
B[*a*]P (N=33)	B[*a*]P	AIN-93G	33 ±5	-	P<0.005
B[*a*]P/2%CG (N=37)	B[*a*]P	AIN-93G with 2%CG	32 ±4	3%	NS
B[*a*]P/4%CG (N=38)	B[*a*]P	AIN-93G with 4%CG	25 ±5	24%	P<0.005

The post-initiation treatment with CG significantly reduced of alveolar and bronchiolar epithelial cell proliferation (Table 2). The percentage of lung cells stained with anti-BrdU 14 weeks after the last dose of B[*a*]P significantly decreased the level of cells stained with anti-BrdU by 69% and 88% in the case of AIN093 diet containing 2% and 4% CG, respectively, compared to the group of mice administered with carcinogen alone. In the present 20-week mouse lung carcinogenesis, CG administered via AIN-93 diet significantly decreased lung hyperplasia and adenoma incidence.

Table 2: Inhibitory effect of dietary calcium D-glucarate (CG) on B[*a*]P-induced alveolar and bronchiolar epithelial cell proliferation in female A/J mice. Values are means ±SD. Statistical analysis was done with the use of Student *t*-test. Both CG groups were compared to the B[*a*]P group.

MICE GROUP (N)	CARCINOGEN	DIET	BrdU LABELED CELLS IN ALVEOLAR/ BRONCHIOLAR REGIONS (%)	INHIBITION %
Control (N=5)	None	AIN-93G	0.28 ± 0.08	_
B[*a*]P (N=5)	B[*a*]P	AIN-93G	5.36 ± 0.75	_
B[*a*]P/2%CG (N=5)	B[*a*]P	AIN-93G with 2%CG	1.64 ± 0.25 (P < 0.0005)	69%
B[*a*]P/4%CG (N=5)	B[*a*]P	AIN-93G with 4%CG	0.64 ± 0.39 (P < 0.0005)	88%

As shown in Figure 1 gradual inhibition of adenoma counts formation by CG dietary took place, i.e. 20% and 44% respectively, however, statistical significance was reached at higher concentration of CG (P<0.005). Interestingly, the effectiveness of CG supplementation in inhibition of mouse hyperplasia/adenoma formation correlated well with its suppressive action on serum β-G.

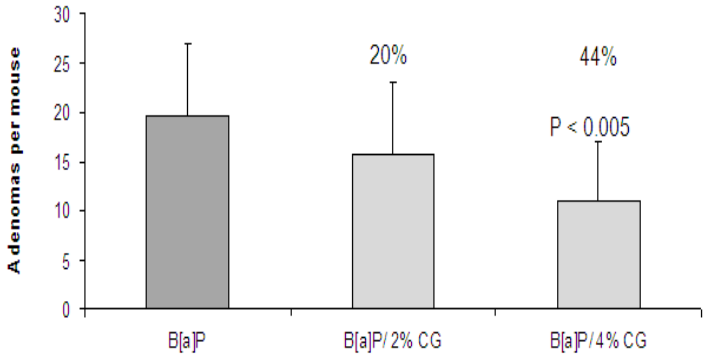


Figure 1: Inhibitory effect of post-initiation dietary calcium D-glucarate (CG) on benzo(*a*)pyrene (B[*a*]P) – induced lung carcinogenesis in female A/J mice. Adenoma counts are means +/- SD.

Anti-inflammatory activity of CG

It has been stated that the proinflammatory molecules such as IL-1 and C-reactive protein (CRP) as well as other inflammation markers correlate with the decrease of β-G activity in patient's sera with inflammation.[29,30] It is generally accepted that inflammatory microenvironment is involved in carcinogenesis. Proinflammatory molecules exerted in response to the different stressors such as infection agents and inflammation and also in response to carcinogens.[31-33] Numerous proinflammatory cytokines are detected in the microenvironment of diverse cancers, and the molecules like TNFα undertaken the transformation of precancerous to malignant cells.[34,35] Previously, we conducted the experiments to examine how CG dietary supplementation of AIN-93 diet in B[*a*]P-induced mouse lung cancer (14-weeks model) influenced on serum inflammatory molecules.[17] The obtained data revealed that dietary CG diminished gradually serum level of TNFα, IL-6 and IL-12p70. On the other hand, proinflammatory cytokine, IL-10 increased its level by about 250 and 288% in the case of 2 and 4% CG diets, respectively, after 4 weeks after second dose of carcinogen. Interestingly six weeks after the second dose of B[*a*]P, the level of IL-10 significance decreased by over 51 and 42% in the case of 2 and 4% CG application, and becoming below detection at 10 weeks after the second dose of B[*a*]P.

In the current report, the activity of cyclooxygenase-2 (COX-2) in B[*a*]P-induced mouse cancer lung was evaluated. As is shown in Figure 2, dietary CG markedly inhibited COX-2 activity. The 2% CG decreased COX-activity by over 30% and the 4% CG diet by over 47% in comparison to the B[*a*]P mice group. COX is the key enzyme in the conversion of arachidonic acid to prostaglandins and other bioactive lipids, involved in several physiological and pathogenic pathways.[36,37]

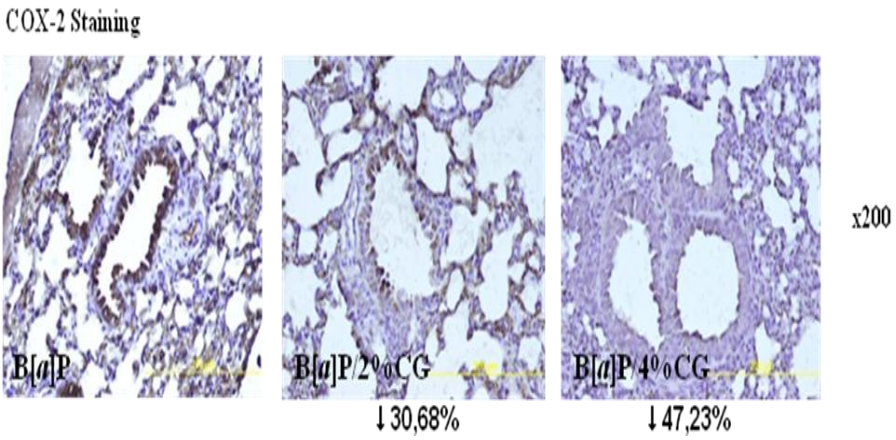


Figure 2: COX-2 expression in B[*a*]P and CD dietary (2 and 4%) lung mice A/J, lung sections immunostained for COX-2, respectively.

Three isoforms of COX have been identified.[38,39] COX-1 is constitutively expressed in most tissues and has been postulated to play the role as a "housekeeping" gene product involved mainly in gastrointestinal track. COX-3, an alternate splice variant of COX-1 has been discovered in canine brain.[40] An inducible isoform – COX-2 is of particular interest because of its association with inflammatory diseases and carcinogenesis.[41] COX-2 is dramatically upregulated by proinflammatory and mitogenic stimuli such as TNFα and other cytokines, growth factors and cancer promoters.[42] Overexpression of this enzyme in lung cancers is associated with progression of the disease and adverse patient outcome.[37,43,44] Its high expression is observed in

premalignant and malignant lesions. The precise mechanism engaged in COX-2 expression in carcinogenesis are not completely clear, however, activity of this enzyme may impact on lung cancer formation since it can activate the pulmonary carcinogenic agents like B[*a*]P.[45]

This inducible COX isoform is detected in several cell types, i.e., macrophages, fibroblasts, cancer cells and "activated" endothelial cells.[41,46] In the present study the numbers of macrophages and proliferating type 2 pneumocytes in the lung A/J mice treated with B[*a*]P and with CG AIN-93 diet were determined. We observed increased numbers of cancer-associated macrophages and type 2 pneumocytes in lung of mice after B[*a*]P treatment compared to control samples (figure 3). Marcophages represented ~22% of total labeled cells, while type 2 pneumocytes ~43% of total labeled cells.[47] The exemplary microphotographs (Figure 3) showed that CG dietary caused markedly and dose-related decrease in the number of macrophages (i.e., up to 44%) and type 2 pneumocytes (i.e., up to 37%) in alveoli of B[*a*]P-treated mice.

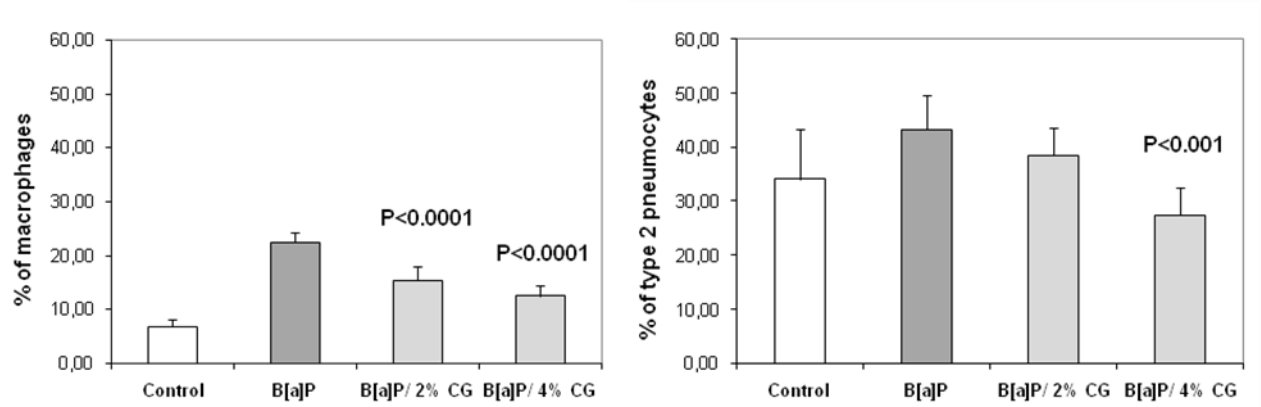


Figure 3: Effect of post-initiation dietary D-glucarate on the percentage of the proliferating type 2 pneumocytes and macrophages in the lung of A/J mice.

The lung represents a very heterogenous organ consisting of over 40 different cell types which are characterized by unique morphology and functions.[48] Alveolar macrophages account for 5% of peripheral lung cells. Their phagocytic activity is important to maintenance of clean and sterile alveoli.[49] These cells take parts in inflammation in alveolar space. Epithelial type 2 pneumocytes account for 12% of the total alveolar cell population. These cells are known to synthesize and secrete of pulmonary surfactant, which reduces surface tension and prevents lung collapse during expiration. They play a key function in xenobiotic metabolism and tissue renewal after lung damage caused by different compounds.[50] The results published by Dusinska et al[49] revealed different responses of rat alveolar macrophages and epithelial type 2 pneumocytes to oxidative damage after treatment with pesticide, paraquat. The authors observed the background level of strand breaks was about five fold higher in type 2 pneumocytes than in alveolar macrophages. A high level of endogenous damage in type 2 pneumocytes suggests that this pulmonary cell type indicating slow DNA repair, is at least consistent with their probable role as progenitors of lung cancer.[49,51]

The inhibition of COX-2 activity by CG in B[*a*]P induced pulmonary hyperplasia could be of importance in lung cancer prevention and management by this natural agent and other (derivatives of GA) especially in spite of data indicating that COX-2 inhibitors have been shown to induce apoptosis in lung carcinoma cells.[44,52-54]

Proapoptotic activity of CG

Cancer can be considered as the results of the series of genetic changes during which normal cell is transformed into a malignant one while evasion of cell death/apoptosis is one of the main reason in cell that provokes this malignant transformation.[55,56] Over forty years ago apoptosis had linked to the removing of malignant cells, hyperplasia and cancer progression.[57] Therefore, main research efforts are focused on the development of novel approach that aims to selectively kill cancer cells while protecting normal ones.[58] Anticancer drugs exert at least part of their cytotoxic activity by triggering apoptosis.[59,60] It is a highly regulated cellular process whereby most damaged, infected, unfunctional, unwanted cells are eliminated what leads to homeostasis.[61,62] The mechanism of apoptosis is complex and involves large number pathways.[63] The central mediator and executioner of apoptotic machinery is a system involving cysteinyl-aspartate proteases named caspases.[64]

We have focused our attention on expression of two apoptosis – related proteins, i.e., cysteinyl-protease, caspase-9 and nuclear enzyme, PARP-1 in mouse lung tissue induced by B[*a*]P and by dietary supplementation with CG. In the figure 4 the lung cell stained with antibodies identifying caspase-9 large subunit with mol.

wt of 37 kDa and PARP-1 (89 kDa subunit) in lung section isolated from mice treated with B[a]P and CG (2 and 4%) supplemented diet are shown, respectively. Post-initiation dietary CG revealed high proapoptotic effect as evidenced by an elevated level of cleaved caspase-9, i.e., over 50 and 99% after mice were fed with 2 and 4% CG, respectively, compared to the B[*a*]P group (Figure 4A). The lung cell number immunostained with PARP-1subunit 89kDa antibodies was increased by over 152 and 132% in the case of 2 and 4% CG diets, compared to the B[*a*]P treated mice (Figure 4B).

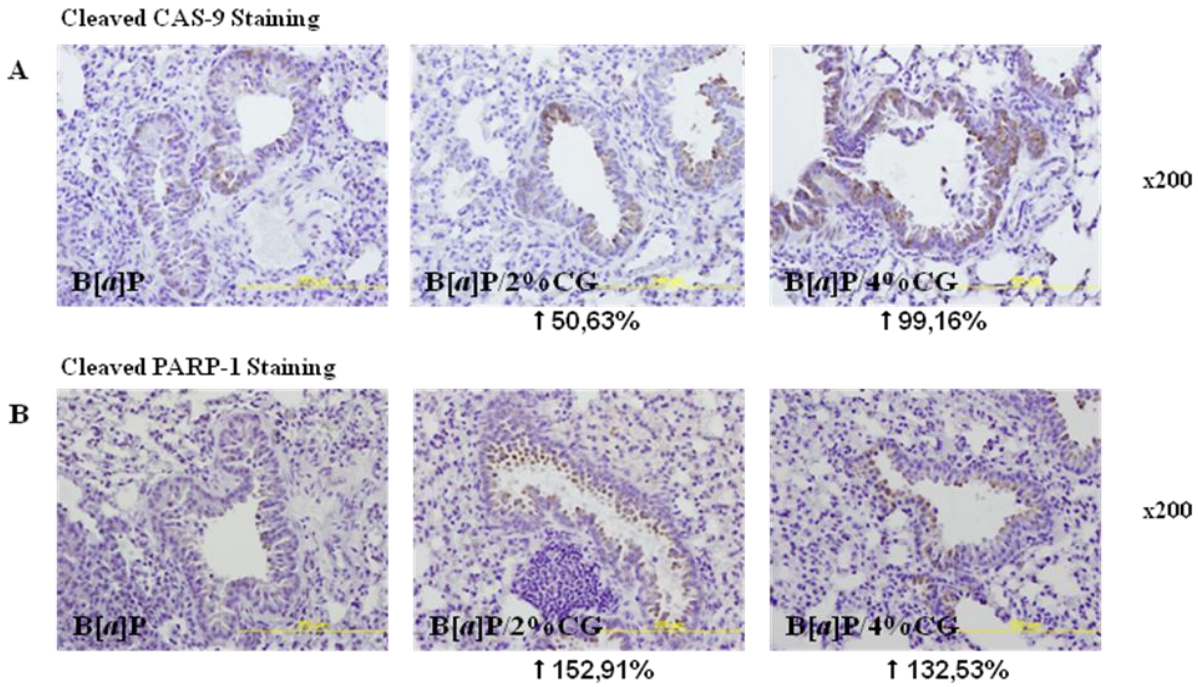


Figure 4: Caspase 9 (37 kDa subunit) and PARP-1 (89 kDa subunit) in lung mice A/J treated with B[a]P or CG (2 and 4%) suplemented diet, lung sections immunostained for caspase 9 (A) and PARP-1 (B), respectively.

Caspase-9 is an apical enzyme triggering intrinsic mitochondrial apoptosis pathway.[65] It is generally accepted that drugs and numerous experimental agents removed blockage of the apoptotic signal from mitochondria which is responsible for apoptosis resistance in cancer cells.[66] Poly (ADP-ribose) polymerase-1 is a 113/116 kDa nuclease enzyme activated by DNA strand breaks and is involved in DNA repair. PARP-1 is one of the first described protein to be cleaved during apoptosis, mainly via caspase-3.[67] The presented in the current report findings show that CG – ß-glucuronidase blocker may act as a potent inhibitor of lung hyperplasia/proliferation, inflammation and it is valuable inducer of apoptosis process. These properties suggest that it may be useful agent in the prevention of lung cancer as well as other cancers.

CONCLUSIONS

Calcium D-glucarate may inhibits promotion and progression of lung carcinogenesis in A/J mice by inhibition of β-G, limiting of cell proliferation, suppression of chronic inflammation, as well as inducingof cell apoptosis. Therefore, the consumption of fruits and vegetables naturally rich in D-glucaric acid derivatives, supplementation and beverages offers promising cancer prevention.

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We would like to dedicate this work in Memory of our Real Friend - Professor Zbigniew Walaszek

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