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Cranberry proanthocyanidins modulate reactive oxygen species in Barrett's and esophageal adenocarcinoma cell lines

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Abstract

BACKGROUND—We recently reported that a cranberry proanthocyanidin rich extract (C-PAC) induces autophagic cell death in apoptotic resistant esophageal adenocarcinoma (EAC) cells and necrosis in autophagy resistant cells. EAC is characterized by high morbidity and mortality rates supporting development of improved preventive interventions.

OBJECTIVE—The current investigation sought to investigate the role of reactive oxygen species (ROS) in the context of C-PAC induced cell death.

METHODS—A panel of human esophageal cell lines of EAC or BE (Barrett's esophagus) origin were treated with C-PAC and assessed for ROS modulation using CellROX[®] Green reagent and the Amplex Red assay to specifically measure hydrogen peroxide levels.

RESULTS—C-PAC significantly increased ROS levels in EAC cells, but significantly reduced ROS levels in CP-C BE cells. Increased hydrogen peroxide levels were also detected in C-PAC treated EAC cells and supernatant; however, hydrogen peroxide levels were significantly increased in medium alone, without cells, suggesting that C-PAC interferes or directly acts on the substrate. Hydrogen peroxide levels did not change in C-PAC treated CP-C BE cells.

CONCLUSION—These experiments provide additional mechanistic insight regarding C-PAC induced cancer cell death through modulation of ROS. Additional research is warranted to identify specific ROS species associated with C-PAC exposure.

Keywords

Cranberry proanthocyanidins; esophageal adenocarcinoma; Barrett's esophagus; ROS; JHAD1; OE33; OE19; CP-C; hydrogen peroxide

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1. Introduction

Numerous health benefits are attributed to cranberry consumption, a small red berry for which over 150 different phytonutrients are described [1]. Cranberry, whether as a juice or extract, imparts urinary tract and cardiovascular health benefits clinically [2, 3]. More recently, preclinical models support cancer inhibitory capacity against numerous cancers including esophageal adenocarcinoma (EAC), ovarian, prostate, breast and lung [4–14] with the strongest *in vivo* effect seen in bladder and colon cancer [15, 16]. The best known health-associated use of cranberries is in the prevention and treatment of urinary tract infections caused by uropathogenic *Escherichia coli* [17, 18]. Flavonoids are one major class of cranberry bioactive components and include anthocyanins, flavonols and proanthocyanidins (PAC). Cranberry's ability to inhibit urinary tract infections is largely attributed to the PAC fraction [1, 19, 20]. Antioxidant effects of these polyphenolic compounds are widely reported and include the ability to decrease lipid oxidation and alter overall markers of oxidative stress [21]. The cranberry proanthocyanidins, also termed C-PAC, are polymers of catechin and epicatechin units with 2–10 degrees of polymerization and at least one or more A-type linkages [1, 2, 21]. The C-PACs are found at fairly high concentration in the cranberry [133–367 mg/100 g fruit; 2].

With respect to cancer, C-PAC is a potent inhibitor of EAC *in vitro* and *in vivo* with a 67.6% reduction in OE19 tumors using a mouse xenograft model [8, 14]. Esophageal cancer is the 7th leading cause of cancer mortality among US males with a 5 year survival rate consistently below 20% [22, 23]. Improved methods for screening, prevention and treatment are needed. Barrett's esophagus (BE), the only identified precursor lesion of EAC, is the result of gastroesophageal reflux disease (GERD) [24–26]. The mechanism of progression from BE to EAC is currently under investigation but likely is multifactorial and characterized by increased genetic abnormalities, including somatic chromosomal alterations preceding cancer [27, 28]. Recently our lab has shown that C-PAC induces autophagic cell death in apoptosis resistant EAC cell lines [8, 14]. Furthermore, autophagy induction was not dependent on Beclin-1, a key regulator of autophagy, in EAC lines [14]. Parallel to *in vivo* xenograft results, C-PAC treatment of EAC cells resulted in downregulation of the PI3K/AKT/mTOR pathway, the central axis for induction of the autophagic cell death pathway.

The association of reactive oxygen species (ROS) and cell death induction is established for cellular necrosis and more recently in the context of autophagy [29]. The overproduction and release of ROS is characteristic of necrotic cell death, while ROS have been shown to regulate autophagy [30, 31]. ROS including superoxide, hydroxyl radical and hydrogen peroxide are generated under conditions of oxidative stress, with increased levels of oxidative damage resulting in activation of cell death pathways [32]. Basal ROS levels in cells act as signaling molecules for growth adaptation and survival. Cancer cells are documented to have higher levels of ROS due to altered metabolic machinery which predisposes cancer cells to increased levels of protein, DNA and lipid damage [29]. Generation of ROS are implicated in the progression of normal cells to cancer cells with cancer cells frequently developing resistance to oxidative stress [33, 34]. A further increase above the heightened basal level of ROS in cancer cells can result in cell death. This in turn

has led to the development of several ROS-inducing drugs including cisplatin (used to treat EAC), cyclophosphamide and fenretidine [35, 36]. Increased ROS levels have been reported in patients with esophagitis and Barrett's esophagus compared to healthy controls and the increased oxidative stress is hypothesized to contribute to carcinogenesis of the esophageal mucosa [37, 38]. Therefore an understanding of the oxidative environment of BE and EAC is important for characterizing the cell death mechanism induced by treatment with C-PAC. ROS is known to be involved in progression to BE and EAC. Two recent reports in ovarian cancer and neuroblastoma reported pro-oxidant effects linked to cell death induction [6, 39]. In this study, we sought to determine the effects of C-PAC on ROS modulation of EAC and BE cells. C-PAC induced generation of ROS in EAC lines with significant increases in hydrogen peroxide observed for JHAD1 and OE19 cells. Conversely, levels of ROS were reduced in C-PAC treated CP-C BE cells.

2. Materials and methods

2.1. Cranberry extract isolation and purification

Cranberry fruit (*Vaccinium macrocarpon* Ait.) was collected at the Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ. Purified C-PAC extract was isolated from cranberries of the 'Early Black' cultivar utilizing solid-phase chromatography as previously described extensively [8, 19, 40–42]. Purified freeze-dried C-PAC was stored at -80°C until used in experiments where it was diluted in treatment medium with a final concentration of 0.001% ethanol.

2.2. Cell culture

Authenticated human BE and EAC cell lines were utilized in this series of experiments. CP-C cells were purchased from ATCC (CRL-4029) and maintained in complete BE growth medium with 5% fetal bovine serum (FBS) as recommended by ATCC. JHAD1 cells were isolated from a distal EAC, stage III, N0 in 1997 (kind gift from Dr. James Eshleman, Johns Hopkins University, Baltimore, MD), OE33 cells were isolated in 1993 from a distal EAC, stage II, N0 (ECACC, Wiltshire, UK) and OE19 cells were isolated in 1993 from an adenocarcinoma at the gastro-esophageal junction, stage III, N1 (ECACC, Wiltshire, UK). EAC cells were grown in RPMI 1640 complete medium containing L-glutamine (2.0 mM), penicillin (10^4 units/mL), streptomycin (10^4 $\mu\text{g/mL}$), sodium pyruvate (1 mM) and 10% fetal bovine serum. All cell culture reagents were from ThermoFisher Scientific (Waltham, MA). Cells were maintained as monolayers at 37°C with 95% air and 5% CO_2 . In all tissue culture assays, the vehicle was 0.001% ethanol diluted in the appropriate medium.

2.3. CellROX[®] green detection of ROS

C-PAC modulation of oxidative stress was measured using CellROX[®] Green reagent, a non-specific indicator of ROS, based on the manufacturer's recommendations (ThermoFisher Scientific, Waltham, MA). When in a reduced state, CellROX[®] Green is a weakly fluorescent cell-permeable dye that exhibits bright green photo stable fluorescence upon oxidation by ROS and subsequent binding to DNA. JHAD1 (10E3 cells/well), OE33 (10E3 cells/well) and OE19 (15E3 cells/well) EAC cells were plated in 96-well black walled, clear bottom plates (Greiner Bio One, Monroe, NC). CP-C BE cells were plated at 10E3 cells/well

in the same plates. Cells adhered for 26–30 h and were treated for 3 or 6 h with vehicle, the ROS inducer *tert*-Butyl Hydroperoxide (TBHP; 100 μ M final concentration; positive control; ThermoFisher Scientific, Waltham, MA) or C-PAC [25 μ g/ml to 100 μ g/ml] alone or in combination with the antioxidant *N*-acetyl-*L*-cysteine (NAC; 5 mM final concentration; Sigma-Aldrich, St. Louis, MO). C-PAC concentrations were selected based on previous studies which determined the LD50 to be in the 50 to 100 μ g/mL range in various cancer cell lines [7, 43, 44]. The concentrations of C-PAC used in these experiments are readily achievable in humans and are already under evaluation for oral and urinary tract health benefits. All treatments were diluted in phenol-red free RPMI complete media with 5% FBS and complete BE growth medium with 5% FBS for EAC and BE cells respectively. CellROX[®] Green reagent was added to the cells and incubated for 30 min at 37°C, subsequently washed once with PBS prior to fluorescent detection using the SpectraMax[®] i3 Multi-Mode Detection Platform. Cellular imaging was done using the SpectraMax[®] MiniMax[™] Imaging Cytometer with excitation/emission wavelengths of 460/535 nm. The data were analyzed using the SoftMax[®] Pro 6.2 Software. A minimum of 4 wells were analyzed for each treatment and expressed as a mean percentage of vehicle treated cells \pm SEM.

2.4. Amplex red hydrogen peroxide assay

Hydrogen peroxide levels were measured following C-PAC treatment of EAC and BE cells using the Amplex Red Hydrogen Peroxide/Peroxidase kit according to the manufacturer's recommendations (ThermoFisher Scientific, Waltham, MA). JHAD1 and OE19 EAC cells were plated at 10E3 and 15E3 cells/well, respectively in 96-well black walled, clear bottom plates (Greiner Bio One, Monroe, NC). CP-C BE cells were plated at 8E3 cells/well in the same plates. Cells adhered for 26–30 h, washed once with PBS and treated for 6 h with vehicle, C-PAC [50–100 μ g/mL], the inducer 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ; 20 μ M final concentration; Sigma Aldrich, St. Louis, MO) and the inhibitor diethylene triamine pentaacetic acid (DTPA; 100 μ M final concentration; Sigma Aldrich, St. Louis, MO). After 6 h, the supernatant was transferred to a new plate and the cells were washed. The cells, the supernatant from the cells and the reaction-no cells in medium were assayed independently for hydrogen peroxide 30 min after addition of the Amplex Red substrate. Fluorescence was measured using the SpectraMax[®] i3 with excitation/emission wavelengths of 545–590 nm. A minimum of 4 wells were analyzed for each treatment and the data expressed as mean relative fluorescence units \pm SEM.

2.5. Statistical analysis

GraphPad Prism software was used to evaluate statistical significance using ANOVA with Tukey's *post-hoc* test. *P*-values of <0.05 were considered significant.

3. Results and discussion

3.1. C-PAC differentially modulates ROS in EAC and BE cells

Excess generation of ROS leads to increased oxidative stress and in turn activates intracellular signaling cascades that can lead to cellular death. Our previous results show that a number of the MAPK proteins differentially altered by C-PAC treatment in EAC cell lines

belong to pathways implicated in hypoxia, ROS generation and altered bioenergetics [8]. In addition, cranberry extracts reportedly increase apoptosis of neuroblastoma and ovarian cancer cells in part through ROS generation [6, 39]. To better understand the cell death mechanism elicited by C-PAC, we examined whether C-PAC induced or inhibited ROS following treatment of esophageal cells. We also evaluated the ability of the antioxidant NAC to mitigate these effects. JHAD1, OE33 and OE19 EAC cells were treated for 3 and 6 h with vehicle, C-PAC [25 and 100 µg/ml], and the known ROS inducer TBHP [100 µM], either alone or in combination with NAC [5 mM]. ROS were detected utilizing CellROX[®] Green fluorescent reagent and the results are presented in Fig. 1A-B. The bar graphs show the percent change in ROS by treatment relative to ROS levels in vehicle treated cells. Numerical representation of the data is located in Table 1.

Following a 3 h treatment, ROS levels were significantly elevated in OE33 (8.1-fold), JHAD1 (6.0-fold) and OE19 (2.8-fold) cells with C-PAC at 100 µg/mL (Fig. 1A). Fluorescent images on the right side of the panel show significantly increased ROS production following treatment. The pro-oxidant TBHP strongly induced ROS in JHAD1 cells (19.8-fold) which subsequently resulted in rapid cell death by 6 h. The antioxidant NAC mitigated TBHP induced ROS levels in JHAD1 cells at 3 h and 6 h. NAC mitigated the levels of ROS produced in OE19 cells at the low dose of C-PAC [25 µg/mL] by 50%, suggesting that NAC can scavenge free radicals produced from cells by C-PAC. At 6 h, C-PAC [100 µg/mL] treatment significantly induced ROS in JHAD1 (10.8-fold), OE33 (6.2-fold) and OE19 (3.4-fold) cells. ROS was produced in JHAD1 cells treated with the combination of NAC and C-PAC [100 µg/mL] only at the 6 h time point (12.1-fold). The low dose of C-PAC [25 µg/mL] increased ROS levels only in OE19 when compared to vehicle. These results support that C-PAC acts as a pro-oxidant in EAC cells by increasing total ROS levels prior to cell death induction. A dose-dependent increase in the inhibition of viability was observed for C-PAC treated JHAD1 and OE19 over a 24–72 h treatment window [14]. The varying levels of ROS induction may be linked to specific cell death pathways activated by C-PAC. JHAD1 and OE33 cells have the highest levels of ROS induced by C-PAC and these cells die through low levels of apoptosis and high levels of autophagy [8, 14]. Conversely, the magnitude of ROS generation is lower in C-PAC treated OE19 cells, which mainly die through necrosis [8]. Additional research is needed to better understand activation of cell death pathways in the context of ROS induction.

We next sought to determine if C-PAC induced death of CP-C BE cells was associated with ROS induction. These premalignant dysplastic BE cells have not been characterized extensively with C-PAC but the LD50 is 100 µg/mL at 72 h post-treatment, a concentration that is behaviorally achievable in humans (Katherine M. Weh and Laura A. Kresty, unpublished data). CP-C cells were treated for 3 or 6 h with the same combinations used for the EAC cells (Fig. 2A-B). C-PAC has little effect on ROS levels at 3 h, regardless of concentration (Fig. 2A). However, when C-PAC is combined with NAC, there is a significant reduction in ROS levels at both doses of C-PAC at 3 h. Following 6 h of treatment, 25 µg/mL and 100 µg/mL C-PAC significantly reduced ROS levels by 44% and 79%, respectively. While NAC alone had no effect on ROS modulation at 6 h, the combination of NAC + C-PAC [25 µg/mL] and NAC + C-PAC [100 µg/mL] resulted in significant reductions in ROS by 66% and 36%, respectively. Thus, NAC added to the ROS reducing effects of C-PAC [25

µg/mL] but not at the higher concentration. Of note, baseline levels of ROS in vehicle treated CP-C cells at 3 h and 6 h appear higher in the BE cells compared to all the EAC lines tested (Fig. 2B). Collectively, these data support that C-PAC acts as an antioxidant in CP-C BE cells by decreasing the total ROS levels; yet, as a pro-oxidant in EAC cells, both leading to cell death downstream.

The literature is divided on the reporting of cranberry juice and extracts as having either antioxidant or pro-oxidant characteristics. Most *in vivo* and clinical trial studies show that cranberries are powerful antioxidants decreasing lipid oxidation, cholesterol and mean arterial blood pressure [45–50]. *In vitro* studies using cancer cell lines show that cranberry juices and extracts can act as both antioxidants [51, 52] and pro-oxidants [6, 39]. The studies that show the antioxidant capacity of cranberry juices and extracts are mostly conducted in the absence of cells, potentially measuring the chemical reaction but with uncertain biological relevance [53–56]. The ability for C-PAC to induce ROS as a potential cell death mechanism in EAC cells is consistent with EAC chemotherapy using cisplatin to further increase ROS levels as a mode of cell death induction. The reduction of ROS in CP-C BE cells by C-PAC suggests that the cell death mechanism may be different in these premalignant cells compared to the EAC cells.

3.2. Hydrogen peroxide levels increase in EAC cells treated with C-PAC

In general, the CellROX[®] green reagent is used to monitor total ROS levels with preference for superoxide, singlet oxygen and hydroxyl radical. To begin identifying the specific ROS induced by C-PAC in EAC cells, we utilized the fluorescence based Amplex Red hydrogen peroxide assay. JHAD1, OE19 and CP-C cells were treated with C-PAC [50–100 µg/mL], 20 µM DMNQ and 100 µM DTPA for 6 h. DMNQ was used as a positive control for the production of hydrogen peroxide and DTPA was used to account for any trace metal interactions with the substrate [57–59]. The treated cells, the supernatant from the treated cells and the reaction-no cells in the medium were analyzed independently for hydrogen peroxide levels. The reaction-no cells is the C-PAC treatment added to the Amplex Red reagents in the absence of any cells.

A significant dose-dependent increase in hydrogen peroxide was observed in JHAD1 cells treated with 50–100 µg/mL C-PAC (Fig. 3A). Only the high dose of C-PAC [100 µg/mL] significantly induced hydrogen peroxide in OE19 cells (Fig. 3B). DMNQ produced a significant increase in hydrogen peroxide from JHAD1 (11.5-fold) and OE19 (3.4-fold) cells as expected. DTPA increased hydrogen peroxide levels in JHAD1 cells incubated with Amplex red but not in the supernatant collected from treated JHAD1 cells. Hydrogen peroxide levels measured from the supernatant of JHAD1 and OE19 cells treated with C-PAC was significantly increased at both concentrations but the magnitude of increase in the supernatant closely mimicked the levels of hydrogen peroxide detected in the reaction-no cells in RPMI medium alone. The high background of the reaction in RPMI media without cells was alarming and supports that C-PAC may be directly interacting with the Amplex Red substrate, resulting in spuriously elevated hydrogen peroxide levels. It is possible that the inherent structure of C-PAC, with multiple vicinal hydroxyl groups, may be generating ROS within the reaction solution or promoting the oxidation of Amplex red to resorufin [1,

60]. Thus, our data supports that C-PAC significantly induces intracellular hydrogen peroxide in EAC cells, but measurements solely focused on the supernatant may be confounded and not reliable.

Hydrogen peroxide levels in CP-C BE cells were unchanged following a 6 h treatment with C-PAC [50 and 100 µg/mL] or DTPA (Fig. 3C). A significant increase in hydrogen peroxide was observed from CP-C cells treated with the positive control DMNQ, although at a lower magnitude than the EAC cells (1.3-fold compared to 3.4- and 11.5-fold in OE19 and JHAD1 cells, respectively). Background levels of hydrogen peroxide were higher in CP-C cells compared to both EAC cell lines, which is in agreement with the increased ROS levels observed in the CellROX[®] green assay (Fig. 2B). Also consistent with the high background observed in the reaction-no cells in RPMI, the reaction-no cells in BE medium accounted for nearly the entire signal measured in the supernatant from the cells, with the exception of DMNQ. These results suggest that hydrogen peroxide is not the specific ROS decreased in CP-C BE cells treated with C-PAC. Hydrogen peroxide is readily broken down to water and oxygen by catalase, an enzyme with significantly higher activity in healthy patients compared to those with esophageal cancer [61]. This may explain why hydrogen peroxide levels increase in EAC lines following C-PAC treatment; yet, do not change in premalignant CP-C BE cells. The antioxidant and pro-oxidant activities of C-PAC in premalignant BE and EAC cancer lines argues that for use in clinical trials, the oxidative environment may dictate the activity of this natural product. The utilization of antioxidants, particularly in the form of supplements at super physiological levels, have not been efficacious and in the case of beta carotene, actually deleterious [62, 63]. Clinical trials utilizing cranberries or cranberry derived products to target cancer are currently lacking; however, other food derived agents rich in polyphenols, such as black raspberries, have reported positive effects in cancer or premalignancy targeting the aerodigestive tract [64–68]. The current study was limited by its *in vitro* nature and the ability of C-PAC to mitigate ROS *in vivo* remains unknown. We are currently investigating the inhibitory mechanisms of C-PAC in a rodent surgical model for EAC. Future studies will be necessary to interrogate additional oxygen radicals for their contribution to C-PAC induced ROS. Finally, examination of reactive nitrogen species including free radical nitric oxide (NO) following C-PAC treatment will be relevant as NO levels are elevated in patients with BE [69].

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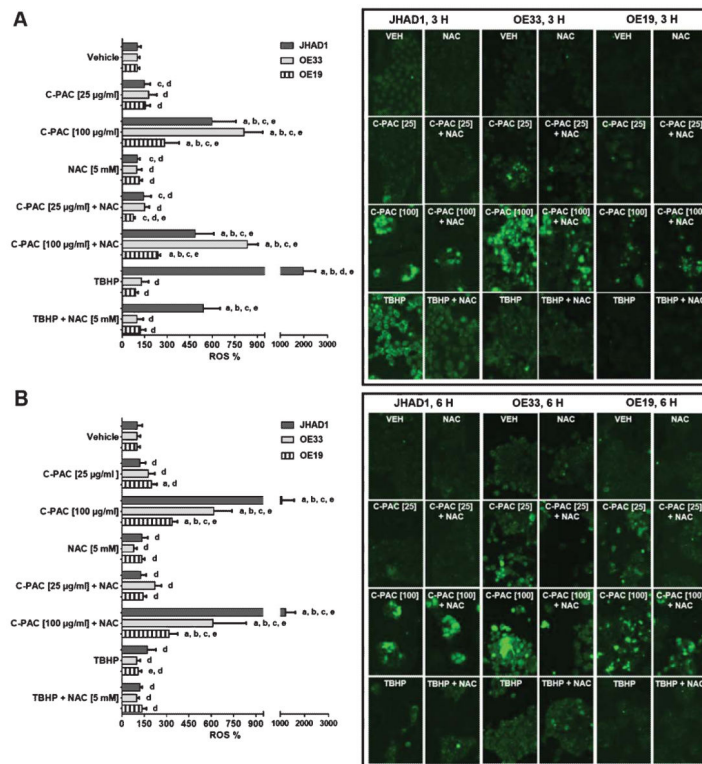
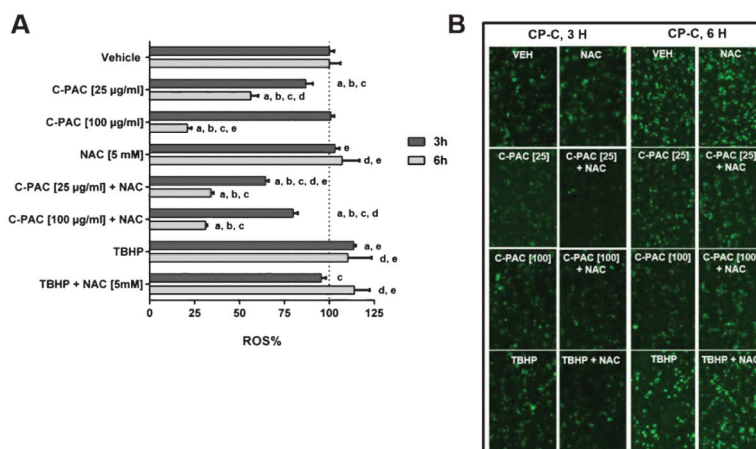
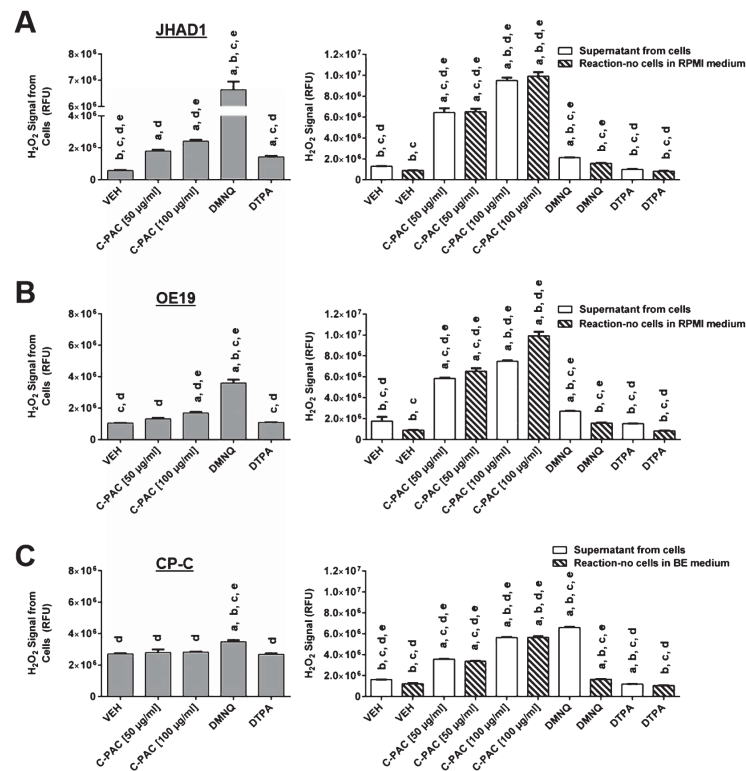


Fig. 1. C-PAC induces ROS in EAC cells. JHAD1, OE33 and OE19 cells were treated for A) 3 h and B) 6 h with vehicle, C-PAC [25 and 100 µg/mL], and the known ROS inducer TBHP [100 µM], either alone or in combination with the antioxidant NAC [5 mM]. ROS induction was detected utilizing CellROX[®] Green reagent coupled with the SpectraMax[®] MiniMax[™] Imaging cytometer as described in the Materials and Methods. Representative fluorescent images are provided where green fluorescent or light grey if viewing image in greyscale signal denotes ROS detection by the CellROX[®] Green reagent. ROS modulation data are expressed as mean percentage of vehicle treated cells ± SEM. Statistical significance was assessed by ANOVA with Tukey's *post-hoc* test ($P < 0.05$). Treatments were significantly different from a = vehicle, b = NAC [5 mM], c = TBHP [100 µM], d = C-PAC [100 µg/mL] and e = C-PAC [25 µg/mL].

**Fig. 2.**

C-PAC reduces ROS in CP-C BE cells. CP-C BE cells were treated for A) 3 h and 6 h with vehicle, C-PAC [25 and 100 µg/mL], and the known ROS inducer TBHP [100 µM], either alone or in combination with the antioxidant NAC [5 mM]. ROS induction was detected utilizing CellROX[®] Green reagent coupled with the SpectraMax[®] MiniMax[™] Imaging cytometer as described in the Materials and Methods. B) Representative fluorescent images are provided where green fluorescent or light grey if viewing image in greyscale signal denotes ROS detection by the CellROX[®] Green reagent. ROS modulation data are expressed as mean percentage of vehicle treated cells ± SEM. Statistical significance was assessed by ANOVA with Tukey's *post-hoc* test ($P < 0.05$). Treatments were significantly different from a = vehicle, b = NAC [5 mM], c = TBHP [100 µM], d = C-PAC [100 µg/mL] and e = C-PAC [25 µg/mL].

**Fig. 3.**

Hydrogen peroxide levels increase or are unchanged in C-PAC treated EAC and BE cells, respectively. The Amplex Red assay was used to measure hydrogen peroxide levels from A) JHAD1, B) OE19 and C) CP-C cells following treatment for 6 h with C-PAC [50 and 100 µg/mL], 20 µM DMNQ and 100 µM DTPA. The supernatant from the cells was transferred to a new plate, the cells were washed once with medium (no FBS) and the reaction-no cells in media were assessed independently for hydrogen peroxide. The reaction-no cells is the C-PAC treatment added to the Amplex Red reagents in the absence of any cells. It was incubated for 6 h at 37°C and 5% CO₂ alongside the plate that contained cells and treatment. Fluorescence was measured as described in the Materials and Methods and data are presented as mean relative fluorescence units ± SEM. Statistical significance was assessed by ANOVA with Tukey's *post-hoc* test ($P < 0.05$). Treatments were significantly different from a = vehicle, b = C-PAC [50 µg/mL], c = C-PAC [100 µg/mL], d = DMNQ [20 µM] and e = DTPA [100 µM].

Table 1ROS induction levels as measured by the CellROX[®] green oxidative stress assay

	JHAD1	OE33	OE19	CP-C
<i>3h Treatment</i>				
VEH	100.0±6.8	100.0±5.5	100.0±6.8	100.0±2.8
C-PAC [25]	147.6±18.6	176.8±22.2	164.8±9.6	86.9±4.0 ^a
C-PAC [100]	579.9±80.3 ^a	813.6±29.1 ^a	295.3±42.0 ^a	100.8±2.0
NAC	98.4±10.2	95.4±10.1	113.4±8.8	103.1±2.6
C-PAC [25] + NAC	154.9±21.2	148.9±18.3	74.1±5.5	64.5±1.8 ^a
C-PAC [100] + NAC	473.4±55.4 ^a	834.4±25.0 ^a	234.6±7.1 ^a	79.8±2.7 ^a
TBHP	2115.1±174.1 ^a	128.2±12.5	85.6±8.4	113.5±1.2 ^a
TBHP + NAC	540.7±69.6 ^a	97.2±17.2	114.3±14.8	95.5±2.7
<i>6h Treatment</i>				
VEH	100.0±11.7	100.0±5.8	100.0±6.9	100.0±6.3
C-PAC [25]	119.4±17.9	177.5±18.0	198.0±14.7 ^a	56.3±4.2 ^a
C-PAC [100]	1079.5±204.7 ^a	617.1±35.2 ^a	337.5±14.7 ^a	21.0±2.4 ^a
NAC	133.3±18.4	79.7±8.6	132.5±9.1	107.1±9.7
C-PAC [25] + NAC	126.3±16.5	220.7±20.7	141.9±10.1	34.2±1.4 ^a
C-PAC [100] + NAC	1214.4±185.0 ^a	610.9±67.8 ^a	323.7±24.9 ^a	31.2±0.9 ^a
TBHP	169.9±23.8	97.2±7.7	108.8±10.3	110.3±13.3
TBHP + NAC	119.0±7.0	98.3±8.9	134.3±13.8	113.9±8.6

Comprehensive tests of statistical significance are reported in Figs. 1 and 2.

^aROS induction levels were calculated for each treatment and are expressed as a mean percentage of vehicle treated cells±SEM. Statistical significance from VEH was assessed by ANOVA with Tukey's *post-hoc* test ($P < 0.05$) and denoted by.