Effect of the novel wound healing agent, OPAL A on leukotriene B_4 production in human neutrophils and 5-lipoxygenase activity

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Abstract

OPAL A is a papaya pulp that is heated and alkalised with bicarbonate (the OPAL process) and is undergoing clinical trials for treatment of chronic wounds. The aim of this study was to investigate possible inhibitory effects of OPAL A and a non-alkalised papaya filtrate on the 5-lipoxygenase signalling pathway. Human isolated neutrophils were incubated with or without OPAL A, non-alkalised papaya or sodium bicarbonate and then exposed to the calcium ionophore, ionomycin to stimulate leukotriene B_4 (LTB_4) production. The production of LTB_4 was inhibited in a dose-dependent manner by all three treatments. The effect of these treatments on 5-lipoxygenase activity, the enzyme involved in the production of precursors of LTB_4 was investigated using a cell-free assay. 5-Lipoxygenase activity was inhibited by OPAL A and non-alkalised papaya, but not bicarbonate. Column chromatography was used to show that the active components within OPAL A were non-proteinaceous polar compounds. The inhibitory effects of OPAL A and a non-alkalised papaya filtrate on 5-lipoxygenase activity and LTB_4 production suggest a possible anti-inflammatory mode of action.

Keywords: papaya, OPAL A, wound healing, 5-lipoxygenase activity, leukotriene B_4.

Introduction

Inflammation is a response to cellular injury and results in the killing of microbial pathogens and tissue destruction. Resolution involves a switch from a pro-inflammatory phase that produces mediators such as leukotriene (LT) B_4 to a proresolving phase where resolvins and protectins are produced[1-3]. Following activation of neutrophils, $[Ca^{2+}]i$ is elevated, initiating the translocation of 5-lipoxygenase to the perinuclear envelope where it interacts with a 5-lipoxygenase activating protein and arachidonic acid to produce LTA_4, the precursor of LTB_4[4,5]. LTB_4 activates BLT_1 and BLT_2 receptors to elicit recruitment and penetrative transmigration of neutrophils from the postcapillary venule, and prolongation of neutrophil survival[6-9]. On the basis of such multifaceted involvement in inflammation, BLT_1 and BLT_2 receptors and the synthetic 5-lipoxygenase signalling pathway have become potential therapeutic targets in the management of conditions in which inflammation is implicated[10-12].

OPAL A is a filtrate that has been manufactured following the homogenisation, heat treatment, alkalisation and filtration of the pulp of the ripened fruit of *Carica Papaya* (the OPAL process), and is currently being examined in a clinical trial conducted by Phoenix Eagle Company in patients who have non-healing wounds (ClinicalTrials.gov Identifier NCT00933348). The mechanisms by which OPAL A contributes to wound healing are not known. We have previously reported a nitric oxide-dependent vasorelaxant effect of OPAL A and a non-alkalised papaya filtrate which raised the possibility that OPAL A might improve perfusion within the wound region[13]. The aim of this study is to examine possible inhibitory effects of OPAL

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A and a non-alkalised papaya filtrate on LTB_4 production and 5-lipoxygenase activity.

Materials and methods

Isolation of human neutrophils

Whole blood was obtained from the antecubital vein of five healthy men (22–55 years) and placed in EDTA tubes. Neutrophils were isolated by differential centrifugation using polymorphprep according to manufacturer's instructions (Axis-Shield, Oslo, Norway). Briefly, whole blood was layered onto polymorphprep in a 1:1 ratio and centrifuged at 500xg for 30 minutes at 22°C in an Eppendorf Centrifuge 5702 with a swing rotor. The fraction containing neutrophils was collected and diluted with 8 ml M199 culture media containing 20% fetal calf serum, 2 mM Glutamax-1, 2.5 µg/ml fungizone and 50 µg/ml penicillin/streptomycin (M199 media), and spun at 450xg for 30 minutes at 22°C. The cell pellet was resuspended in 450 µl Hanks' Balanced Salt Solution (HBSS), with 10 µl of sample smeared onto a microscope slide and stained using Diff Quik differential dye to confirm successful isolation of neutrophils (Figure 1A). This study was conducted in 2010–2011 and conforms to the Statement on Human Experimentation and was carried out with approval from the Human Research Ethics Committee of the University of the Sunshine Coast (A/10/243).

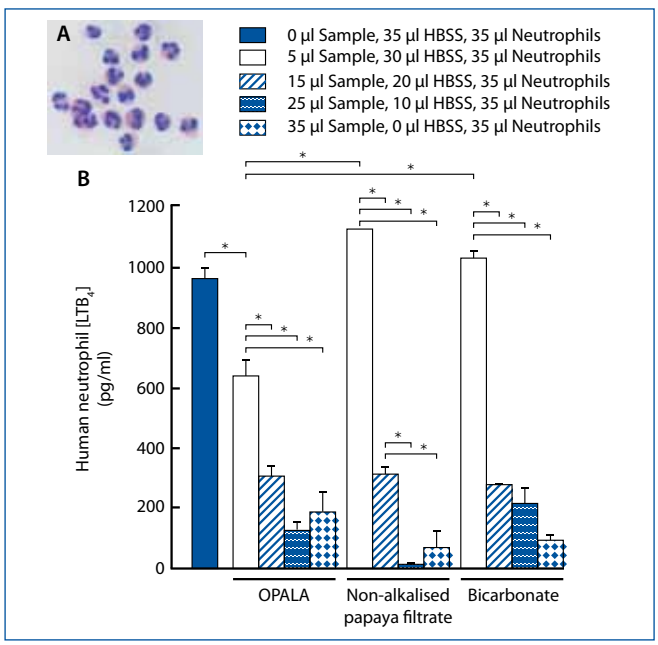

*Figure 1. Effect of OPAL A, non-alkalised papaya filtrate and sodium bicarbonate on ionomycin-stimulated production of LTB_4 in isolated human neutrophils. Successful isolation of neutrophils was achieved by differential centrifugation, as indicated by staining using Diff Quik differential dye (A). OPAL A was more potent than non-alkalised papaya and sodium bicarbonate for inhibition of LTB_4 production (B). Values are mean±SEM, n=4-5, *, P<0.05.*

Effect of OPAL A and non-alkalised papaya filtrate on LTB_4 concentration in ionomycin-stimulated human neutrophils

Aliquots of resuspended cells (35 µl) were combined with OPAL A, non-alkalised OPAL A or 10% bicarbonate (5-35 µl), with solutions prepared to a final volume of 70 µl with HBSS. Samples were incubated for 20 minutes at 37°C in a 5% CO_2 incubator, and then incubated for a further 5 minutes at 37°C with 2 µM ionomycin before centrifugation at 40xg for 6 minutes at 22°C in a MiniSpin Plus centrifuge (Eppendorf). LTB_4 concentration was determined spectrophotometrically at wavelength 405 nm using 50 µl aliquots of the supernatant in a LTB_4 enzyme immunoassay according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, USA). Background absorbance was measured in the absence of OPAL A, non-alkalised papaya filtrate or bicarbonate and this was subtracted from all readings.

Effect of OPAL A and non-alkalised papaya filtrate on 5-lipoxygenase activity using a cell-free assay

The effect of OPAL A and non-alkalised papaya filtrate on lipoxygenase activity was determined using a modified method of Anthon and Barrett (2001)[14]. Lipoxygenase enzyme (3 µg/ml) was incubated with 10 mM 3-(dimethylamino) benzoic acid, 0.05 M $Na_2HPO_4.12H_2O$ (pH 6.0), 0.5 mM linoleic acid and either OPAL A, non-alkalised papaya filtrate or bicarbonate (100 µl/0.5 ml solution) (Solution A) for 5 minutes at 22°C. Solution B (0.5 ml), containing 10 mM 3-methyl-2-benzothiazolinone and 0.1 mg/ml haemoglobin was added to 0.5 ml of Solution A, and incubated for a further 5 minutes at 22°C. An aliquot of the mixture was measured using a spectrophotometer at 598 nm.

Column fractionation of OPAL A

To further characterise the inhibitory activity of OPAL A, a 1.5 ml aliquot of OPAL A was passed through a 0.45 µm syringe filter and the filtrate (0.6 ml) was loaded onto an OASIS column that was pre-activated by application of methanol then distilled water. The column was eluted using a mixture of methanol and water (1:1), and the eluant was collected and analysed using the 5-lipoxygenase activity assay described above. In a second series of experiments, 7.0 ml of OPAL A was mixed with 5 g of silica gel. The sample was frozen at –80°C, freeze-dried, then added to the top of a column containing 5 g silica gel that was moistened with ethyl acetate. The column was eluted with successive rinses with 40 ml of ethyl acetate, acetone, methanol and water. The ethyl acetate, acetone and methanol fractions were recovered by evaporation of solvent at 22°C under a stream of nitrogen. The aqueous fraction was freeze-dried. Samples were reconstituted in 0.5 ml phosphate buffer solution (pH 9) and analysed using the lipoxygenase activity assay as described above.

Statistics

Data were compared using one way Analysis of Variance with Tukey's Honestly Significant Difference (HSD) test using IBM SPSS Statistics Version 19. Data are expressed as mean±SEM.

Results

Effect of OPAL A and non-alkalised papaya filtrate on leukotriene B_4 production in ionomycin-activated neutrophils

OPAL A, non-alkalised papaya filtrate and sodium bicarbonate inhibited the ionomycin-stimulated production of LTB_4 in isolated human neutrophils. The lowest amount of OPAL A used in the assay (5 µl / 35 µl of cells) caused a significant reduction in LTB_4 production compared to an untreated control, and this was maximal for 15 µl of OPAL A/35 µl of cells (Figure 1B). The same amount of non-alkalised papaya filtrate or 10% sodium bicarbonate did not inhibit LTB_4 production. Significant inhibition of LTB_4 production was observed using 15 µl of non-alkalised papaya or 10% sodium bicarbonate / 35 µl of cells, and this was maximal with 25 µl of non-alkalised papaya or 15 µl of 10% sodium bicarbonate /35 µl of cells.

Effect of OPAL A and non-alkalised papaya filtrate on 5-lipoxygenase activity

OPAL A and the non-alkalised papaya filtrate produced concentration-dependent inhibition of 5-lipoxygenase activity. The equivalent amount of sodium bicarbonate to that contained within each OPAL A solution had no effect on 5-lipoxygenase activity (Figure 2A). Further characterisation of the 5-lipoxygenase activity was achieved by loading OPAL A onto an OASIS column and eluting it with a 1:1 mixture of methanol and water. The dried and reconstituted eluant retained the capacity for inhibition of 5-lipoxygenase activity that was observed in the non-fractionated OPAL A sample (Figure 2B). A further experiment eluted OPAL A from a silica gel column using successive rinses with ethyl acetate, acetone, methanol, and water. The reconstituted eluants obtained using ethyl acetate and acetone had no inhibitory effect on 5-lipoxygenase activity whereas inhibitory activity was obtained with the reconstituted eluants obtained using methanol and water (Figure 2C).

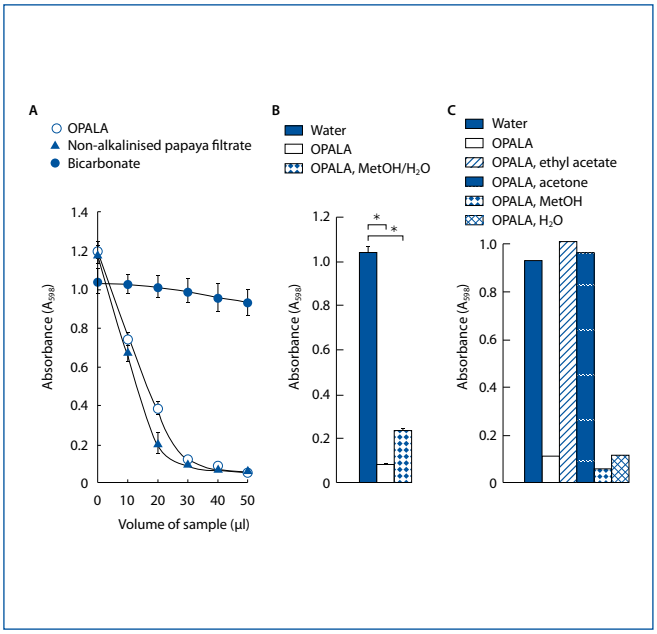

*Figure 2. Effect of OPAL A, non-alkalised papaya filtrate and sodium bicarbonate on 5-lipoxygenase activity in a cell-free assay (n=3). OPAL A and non-alkalised papaya inhibited 5-lipoxygenase activity in a dose-dependent manner, whereas sodium bicarbonate was without effect (A). OPAL A was loaded onto an OASIS column and eluted with methanol/water (1:1) (n=3; duplicate samples). The dried and reconstituted (phosphate buffer solution) eluant inhibited 5-lipoxygenase activity (B). OPAL A was mixed with silica gel and loaded onto silica gel columns and eluted with sequential washes with ethyl acetate, acetone, methanol, and water (n=1, duplicate samples). The dried and reconstituted (phosphate buffer solution) eluants were either without effect (ethyl acetate and acetone eluants) or inhibited 5-lipoxygenase activity (methanol and water eluants) (C). Values are mean±SEM (A, B), *, P<0.05.*

Discussion

Papaya latex harvested from unripe papaya fruit stimulates a pro-inflammatory response when injected into rat paw[15]. In this study we examined the possible anti-inflammatory effects of non-alkalised papaya and papaya that was prepared by homogenisation, heat treatment, alkalisation and filtration of the pulp of ripened papaya (the OPAL process). To our knowledge, this is the first report identifying the inhibitory action of papaya-based filtrates on the 5-lipoxygenase – LTB_4 signalling pathway.

OPAL A, non-alkalised papaya and bicarbonate alone inhibited production of LTB_4 by human neutrophils that were exposed to ionomycin. Significant inhibition of LTB_4 production occurred at a lower concentration of OPAL A than for either the non-alkalised papaya or the equivalent amount of bicarbonate. Since OPAL A contains both papaya and sodium bicarbonate, this finding suggests a possible additive inhibitory action on LTB_4 production. LTB_4 is an integral eicosanoid in the inflammatory response, with roles in recruitment and activation of leukocytes, and prevention of leukocyte apoptosis[7]. Thus, inhibition of LTB_4 production by OPAL A suggests a possible mode of action for this filtrate in the treatment of inflammatory conditions.

The endogenous pathway for the synthesis of LTB_4 from arachidonic acid is well described[4]. 5-Lipoxygenase activity is crucial to the production of LTB_4, first catalysing the oxidation of arachidonic acid to generate 5-hydroperoxyeicosatetraenoic acid, then the subsequent production of LTA_4. LTA_4 is in turn converted to LTB_4 in the presence of LTA_4 hydrolase[4]. In the present study, we showed that OPAL A and non-alkalised papaya inhibited 5-lipoxygenase activity with similar potency. Interestingly, sodium bicarbonate was without effect, contrasting with the ability of bicarbonate ions to inhibit LTB_4 production in the activated neutrophils. The findings indicate that bicarbonate, or the elevation of pH resulting from the addition of bicarbonate, may have a direct suppressive effect on the neutrophils.

OPAL A was eluted from an OASIS column or silica gel column as a preliminary analysis of the chemical properties of the filtrate for inhibition of 5-lipoxygenase activity. The eluant obtained from the methanol/water rinse of the OASIS column retained inhibitory activity that was detected in the non-fractionated OPAL A. The polar solvent mixture (methanol/water) was used to selectively elute polar molecules. When OPAL A was eluted on a silica gel column using non-polar solvents (ethyl acetate or acetone), inhibitory activity was lost. However, elution with polar solvents (methanol or water) retained activity that was detected in the non-fractionated OPAL A. Proteins efficiently adsorb to silica gel[16], so we conclude that the active components within OPAL A are non-proteinaceous, polar molecules. Bioactivity-guided fractionation experiments could be carried out in the future to elucidate the identity of the active compound(s) present in OPAL A. Phenolic compounds such as quercetin and caffeic acid are candidates as they are 1) polar 2) are expressed in *Carica Papaya*[17], and inhibit 5-lipoxygenase activity (IC_{50} for quercetin, 0.6 µM[18]; IC_{50} for caffeic acid, 25 µM[19]) and LTB_4 production in leukocytes (IC_{50} for quercetin, 2 µM[20]; IC_{50} for caffeic acid, 200 µM[21]).

In conclusion, this study has identified an inhibitory effect of OPAL A and non-alkalised papaya filtrate on LTB_4 production and 5-lipoxygenase activity. The findings provide a potential mechanism by which OPAL A might contribute to the treatment of conditions in which inflammation is implicated, such as the treatment of wounds.

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Conflict of interest

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