

THE INFLUENCE OF TWO SYNTHESISED PROSTAMIDES ON OXIDATIVE STRESS INDUCED IN RAW264.7 MACROPHAGE MURINE CELL LINE

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Abstract

Prostamides are lipid mediators biosynthesised via COX-2 from anandamide (arachidonylethanolamide). The aim of this study was to determine the possible toxicity of prostamides after their peroxidation on RAW264.7 macrophage murine cell line. In the presence of 10 μ M H₂O₂ and the studied prostamides, in RAW 264.7 macrophage murine line an oxidative stress occurred, demonstrated by the increase of malondialdehyde (MDA) and protein oxidation levels in a time dependent manner.

Rezumat

Prostamidele sau prostaglandin-etanolamidele sunt mediatori lipidici biosintetizați din anandamidă (arahidonoiletanolamidă). Scopul studiului a fost determinarea unui posibil efect toxic al prostamidelor în urma efectului peroxidant al acestora asupra liniei celulare de macrofage murine RAW264.7. În urma tratării celulelor cu peroxid de hidrogen 10 μ M și prostamidele nou sintetizate, s-a înregistrat, la nivelul liniei de macrofage murine RAW264.7, creșterea nivelului de malondialdehidă (MDA) și al peroxidării proteice într-o manieră dependentă de timp.

Keywords: prostamides, oxidative stress, lipid peroxidation.

Introduction

Prostamides are lipid mediators biosynthesised via COX-2 from anandamide (arachidonylethanolamide), which is a lipid transmitter generated by neurons in the brain [7] as well as by macrophages in a lipopolysaccharide induced manner [6]. Anandamide is an endogenous cannabinoid which is specifically recognized by two distinct G-protein coupled membrane receptors, CB1 and CB2, that modulate effector enzymes such as adenylate cyclase, mitogen activated protein kinases and

members of the nuclear factor κ B family as well as cell membrane ion channels [21].

Previous studies proved that 1 μ M anandamide decreased calcium current in HEK293 cells [23]. Ca^{2+} overload is detrimental to mitochondrial function and can cause mitochondrial reactive oxygen species (ROS) generation [17]. As a result, it could be considered that anandamide has an antioxidant effect in cells. In spite of this, it appeared that depending on cellular type, the effects of anandamide were controversial. So, this cannabinoid reduced the gastric mucosal oxidative stress in rats [24], but increased susceptibility to oxidative stress-induced hepatocyte damage [2] and raised the intracellular superoxide levels in PC-12 cells [20].

In several pathologies, including glaucoma, the generation of reactive oxygen species was considered the base of pathogenesis [9].

At the ocular level, the oxidative damage produces molecular modifications with toxic potential involved in the generation of cataract, glaucoma and neurodegenerative diseases [16]. In normal physiological conditions, the aqueous humour contains low concentrations of hydrogen peroxide and superoxide anion [22]. The photochemical reactions and increased concentration of oxidants expose the trabecular meshwork to an increased level of oxidative stress [9].

Recent *in vitro* studies demonstrated the importance of prostamides (prostaglandin-ethanolamides) in the protection of ocular tissues against the oxidative stress damages and in decreasing the intraocular pressure [8, 26]. It seems that the double bonds present in the chemical structure of prostamides make them susceptible to oxidation in case of increased or chronic exposure of the cell to oxidative stress representing a potential source of peroxides within the cells.

The present study aims to demonstrate the effect of two synthesised prostamides after their peroxidation on RAW264.7 macrophage murine cell line. The oxidative stress biomarkers: malondialdehyde, reduced glutathione, thiol and carbonyl protein groups, as well as advanced oxidation protein products (AOPP) were assayed.

Materials and Methods

All chemicals and reagents were of analytical purity and purchased from Sigma (St. Louis MO) and Gibco. D-Cloprostenol-1-N-ethylamide (PM1) and D-Cloprostenol-1-N- ethanolamide (PM2) were obtained by stereo controlled synthesis within the Department of Biosynthesis of Natural Products from the National Institute for Chemical and Pharmaceutical Research and Development – I.C.C.F., Bucharest, Romania.

Cell Culture and treatment

Murine macrophage RAW 264.7 cells were grown in Minimum Essential Medium with Earle's salts (MEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and ampicillin in a humidified atmosphere (5% CO₂, 95% air) at 37 °C. The cells were exposed simultaneously to 10 µM hydrogen peroxide and prostamides in different concentrations: 100 µM, 200 µM, 300 µM, 400 µM for 24, 48 and 72 hours. For each experiment and time interval, two controls were used i.e. treated with 10 µM hydrogen peroxide solution and an untreated one.

Cell Viability (MTT Test)

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the manufacturer's instructions [14].

Oxidative stress biomarkers

Glutathione Assay

The cell lysate was deproteinized with sulfosalicylic acid 5% and then the reduced glutathione was analyzed using a Sigma kit, following manufacturer's instructions [1]. The yellow product, 5-thio-2-nitrobenzoic acid (TNB) was measured spectrophotometrically at 412 nm. The GSH level was calculated as nmols/mg protein.

Determination of malondialdehyde (MDA)

The concentration of MDA was determined according to del Rio [5]. Cultured RAW 264.7 cells were previously homogenized in one of 0.1M saline phosphate buffer and sonicated on ice, at 40V, 3 times of 30 seconds each. A sample of 3 mg/mL protein was used to determine MDA concentration using 1,1,3,3-tetramethoxy propane as standard. The results were expressed as nmols of MDA/mg protein.

Detection of protein carbonyls

The protein carbonyls were assayed according to Levine's method [10]. The appropriately diluted cell lysates were mixed with 10mM 2,4-dinitrophenylhydrazine (DNPH) in 2mM HCl and then treated with 20% trichloroacetic acid. The absorbance of protein carbonyl was determined in 1M NaOH dissolved pellet at 370nm. The concentration was calculated using the molar absorption coefficient of 22,000 M⁻¹cm⁻¹ and expressed in nmols/mg protein.

Protein thiol groups assay

The protein thiols concentration were assayed according to the method of Riener [19] using N-acetyl cysteine as standard. The values were expressed as nmols/mg protein.

Advanced oxidation protein products assay

The concentration of advanced oxidation protein products (AOPP) was assessed according to the method of Witko-Sarsat [25] using a chloramines-T standard curve.

The protein concentration

The protein concentration was measured according to Bradford method [3] using bovine serum albumin as standard.

Statistical Analysis

All values were expressed as means of triplicate experiment $\pm$ standard deviations (SD). Statistical significance was calculated using t-student test. The results were considered significant if the p value was less than 0.05.

Results and Discussion

Prostamides (prostaglandin-ethanolamides) are local hormones with a similar chemical structure and biosynthesis to prostaglandines. Despite the similarities between the two classes of lipid mediators, the biological role of prostamides is almost unknown. The discovery of bimatoprost, a synthetic prostamide considered nowadays the best antiglaucoma drug, attracted the attention on this class of compounds. Present researches are limited to the ocular level, but less is known about their biological activity on other tissues or cell lines such as macrophages.

Macrophage is a widely distributed cell type in animal organisms including ocular tissues. The RAW264.7 macrophage murine cell line expresses endogenous prostamides but their biological role and the effects in the presence of oxidative stress are not yet elucidated [18].

The synthetic prostamides used in this study have similar structure with the endogenous prostaglandin-ethanolamides.

The influence of the prostamides on the cell viability (MTT test)

The cellular viability test or MTT test demonstrated that the mitochondrial enzymatic activity of macrophages was inhibited in a dose-dependent manner after 24, 48 and 72h of treatment (Figure 1).

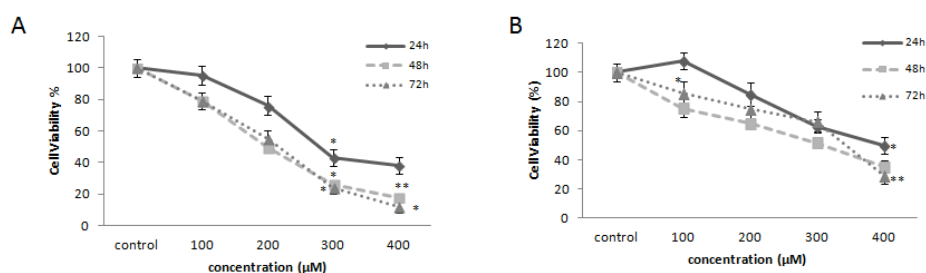


Figure 1

Evaluation of cell viability with (A) D-cloprostamol-1-N-etanolamida and (B) D-cloprostamol-1-N-ethylamide on Raw 264.7 cell line after 24, 48 and 72 hours at concentrations of 100, 200, 300 and 400 μM. The results were expressed relative to control cells and were represented as mean ± SD (n=3). Data were analyzed by Student's t-test. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 compared to control

Analysing Figure 1, it can be seen that the 100 μM concentration of both compounds did not affect significantly the cells viability during 72 hours exposure. The highest decrease of viability was registered in the case of treatment with the dose of 400 μM for both prostamides.

The effect of D-cloprostamol-1-N-ethylamide (PM1) treatment demonstrated a slightly increased toxicity on RAW 264.7 cells compared to D-cloprostamol-1-N-etanolamida (PM2). In the case of 400 μM PM1 treatment, the cell viability significantly decreased by 60% and 88% after 24h and 72h respectively, whereas for the same dose of PM2 it diminished by 50% and 70% at 24h and respectively 72h.

Taking into account these results, the concentration of 100 μM prostamides has been chosen for further studies. This presented a low cellular toxicity and also, it was well correlated with the therapeutic dose used for the treatment of high ocular pressure (~70 μM).

The influence of the prostamide treatment on the malondialdehyde (MDA) level

Malondialdehyde (MDA) is an end product of lipid peroxidation of polyunsaturated fatty, with cellular toxic potential, having atherogenic and mutagenic effects due to the interactions with DNA and free protein amino groups especially of lysine [12, 15].

The treatment of cells with 10 μM H₂O₂ and PM1 or PM2 which generated peroxidized prostamides increased the MDA level at 48h and 72h after the treatment, by about 20% compared to control. In the absence of hydrogen peroxide, MDA concentration increased by approximately 37%, after 48 hours compared to control and after 72 h reach the control level.

Table I

The relative values of malondialdehyde and reduced glutathione after 24, 48 and 72h treatment of RAW 264.7 murine macrophage cell line with 100 μ M D-cloprostenol-1-N-ethylamide (PM1) and 100 μ M D-cloprostenol-1-N-ethanolamide (PM2) in the absence of an oxidant and in the presence of 10 μ M H₂O₂

	Time intervals (hours)	Control	PM1	PM2	H ₂ O ₂	PM1+ H ₂ O ₂	PM2+ H ₂ O ₂
MDA (nmols /mg)	24	100 $\pm$ 7	112 $\pm$ 10	92 $\pm$ 10	100 $\pm$ 13	103 $\pm$ 11	114 $\pm$ 11
	48	100 $\pm$ 14	138 $\pm$ 6	137 $\pm$ 2	100 $\pm$ 8	121 $\pm$ 9	124 $\pm$ 7
	72	100 $\pm$ 5	106 $\pm$ 13	100 $\pm$ 9	100 $\pm$ 2	120 $\pm$ 3	120 $\pm$ 10
GSH (nmols /mg)	24	100 $\pm$ 5	67 $\pm$ 2*	155 $\pm$ 9**	100 $\pm$ 7	75 $\pm$ 12	135 $\pm$ 11**
	48	100 $\pm$ 3	78 $\pm$ 13*	92 $\pm$ 6	100 $\pm$ 11	54 $\pm$ 10*	73 $\pm$ 6**
	72	100 $\pm$ 2	88 $\pm$ 14*	90 $\pm$ 13	100 $\pm$ 14	88 $\pm$ 3	118 $\pm$ 3

Data were analyzed by Student's t-test. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 compared to control.

The PM1 treatment significantly decreased GSH level compared to control at all-time intervals, whereas PM2 increased significantly this parameter after 24 h and decreased after 72h to the control levels. The same pattern was registered in the case of hydrogen peroxide and prostamides simultaneous exposure. A good correlation between MDA and GSH levels can be noticed.

The influence of the prostamides treatment on oxidative modifications of proteins

Protein thiol groups modification is a reversible post-translational process important in redox cell signalling [13]. In the case of non-oxidative treatment, PM1 and PM2 decreased the protein thiols level in the same manner by about 15-17% and 19% after 24h respectively 48h. After 72h, its level increased almost to the control level in case of 100 μ M PM1 and PM2 treatment (Figure 2). In the case of simultaneous exposure to 10 μ M H₂O₂ and 100 μ M PM1 or PM2 a decrease of 38% of this parameter level in comparison with control was registered, after 24h. At higher exposure periods, the protein thiol levels were lower by 22% for PM1 and 18% for PM2 after the second day, and by 18% for PM1 after the third day compared to control. But, after 72h, the decrease induced by peroxidised PM2 in RAW 264.7 cells was not significant compared to control.

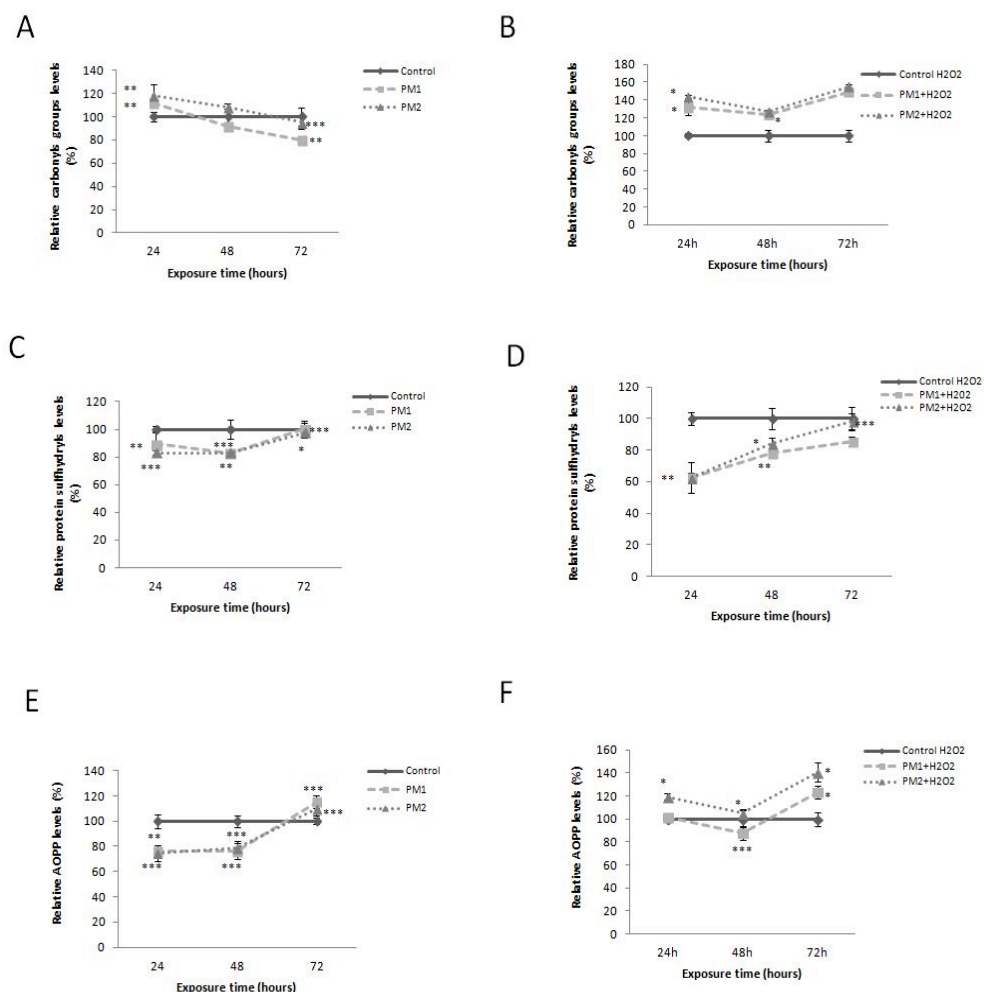


Figure 2

The relative values of protein reactive carbonyl groups (PRCG) (A, B), protein thiols level (C, D) and advanced oxidation protein products (AOPP)-(E, F) after 24, 48 and 72h treatment of RAW 264.7 murine macrophage cell line with 100μM D-cloprosteno-1-N-ethylamide (PM1) and 100μM D-cloprosteno-1-N-ethanolamide (PM2) in the absence of an oxidant (A, B, C) and in the presence of 10μM H₂O₂ (D, E, F). The results were expressed relative to control cells and were represented as mean ±SD (n=3). Data were analyzed by Student's t-test. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 compared to control

Carbonyl groups in proteins may be generated by ROS-mediated oxidation of side chains of some amino acid residues (especially proline, arginine, lysine, threonine) or by glycation and reactions with lipid peroxidation products [4]. They were observed in case of rheumatoid

arthritis, diabetes mellitus, chronic renal failure, respiratory distress syndrome and Alzheimer's disease.

The 24 hour prostamides treatment increased the protein carbonyl groups compared to control (Figure 2). Later on, this increase has diminished and was insignificant compared to control.

In presence of $10\mu\text{M H}_2\text{O}_2$, the oxidized prostamides treatment had a significant pro-oxidant effect with an increased level of protein carbonyls during all time intervals compared to H_2O_2 control. A significant increase of 48% and 50% was noticed at 72h in case of PM1 and PM2 respectively.

Advanced oxidation protein products (AOPP) are uremic toxins which appear within the cell by a chlorination reaction between some oxidants (e.g. chloramines, hypochlorous acid) and plasmatic or tissue proteins. Increased levels of AOPP were noticed in case of chronic renal failure, atherosclerosis, diabetes mellitus, sclerosis and HIV infections [11].

In our experiment, AOPP level decreased significantly by 25% at 24h and 48h after both prostamide treatments compared to control, but increased after 72h by 15% and 10% in case of PM1 and PM2 respectively. In the presence of $10\mu\text{M H}_2\text{O}_2$, the oxidized prostamides produced different effects in a time dependent manner in the case of this biochemical parameter. At 48 h after the treatment with oxidized PM1, the AOPP level was decreased by 13 % followed by a 23% increase at 72h compared to cells treated with $10\mu\text{M H}_2\text{O}_2$. The oxidized PM2 treatment increased the AOPP level by 20% and 40% after 24h respectively 72h in comparison with cells treated with $10\mu\text{M H}_2\text{O}_2$.

Conclusions

In the presence of $10\mu\text{M H}_2\text{O}_2$ and PM1 and PM2, in RAW 264.7 macrophage murine line an oxidative stress occurred, demonstrated by the increases of MDA and protein oxidation levels in a time dependent manner.

The prostamide treatment in the absence of an oxidant had no significant effect on the studied oxidative stress parameters in the RAW 264.7 murine macrophage cell line. The exposure of prostamides to an increase concentration of an oxidant could initiate their peroxidation and contribute to the oxidative stress damage within the cells.

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