ACTA BIOLOGICA CRACOVIENSIA Series Zoologia 57: 61–67, 2015

Resolvin D2 Plays a Protective Role in Raw 264.7 Cells Treated with Polycyclic Aromatic Hydrocarbons

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Accepted October 8, 2015

Eicosanoids are signaling molecules that control the immune processes and might have effects on inflammatory diseases. The aim of our study was to evaluate the effect of added resolvin D2 (RvD2), after treatment with polycyclic aromatic hydrocarbons (PAHs), on RAW 264.7 cells by using a UHPLC/MS-TOF method for the quantification of eicosanoids: 8-iPGF3α, PGF3α, 8-isoPGF2α, PGF2α and 5-iPF2α as well as cyclooxygenase 2 (COX-2), prostaglandin E synthase (cPGES) and prostaglandin F2α (FP) receptor protein expression by Western blot. The levels of PGF3α, PGF2α 8-iPGF3α 8-isoPGF2α and 5-iPF2α were decreased in RAW 264.7 cells after the exposure to PAHs and treatment with RvD2. It was observed that COX-2, cPGES and FP-receptor expression was decreased after co-treatment of the cells with PAHs and RvD2. Our findings suggest that RvD2 has anti-oxidant, anti-inflammatory and pro-resolving properties that may contribute significantly to alleviation of the harmful effects caused by PAHs in macrophages. Moreover, these results suggest that a diet rich in n-3 fatty acids might be helpful in resolving the inflammation and mitigating the effects of environmental stress in macrophages.

Key words: Resolvin D2, macrophage RAW 264.7, polycyclic aromatic hydrocarbons, isoprostanes, FP receptor, COX-2

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PL ISSN 0001-530X

INTRODUCTION

Macrophages are essential in physiological processes including the regulation of innate and adaptive immunity, but they are also important in pathological and inflammatory states. These immune cells possess remarkable plasticity and display various shades of functionalities based on changes in the surrounding molecular environment (Korns et al., 2011). Macrophages can respond to danger signals through upregulation of proteins and synthesis of eicosanoids and other lipid molecules that altogether act to recruit other immune cells to a site of inflammation (Norris and Dennis, 2012). Additionally, macrophages possess the ability to promote tissue repair once infection has been thwarted (Korns et al., 2011).

Eicosanoids are specific biomarkers of inflammation. Their biosynthesis from polyunsaturated fatty acids can be catalysed by cyclooxygenase (COX-2), lipoxygenases (LOX), and cytochrome P450 enzymes (Serhan and Petasis, 2011; Serhan, 2014). Eicosanoids are potent lipid mediators of inflammation and are known to play an important role in numerous pathophysiological processes (Groeger et al., 2010; Lee et al., 2013; Serhan, 2014).

Eicosanoid metabolism has been well characterized using purified enzymes and overexpression studies. Currently, most interest in the field has shifted to defining the pathway in cellular inflammatory contexts. The understanding of the complex networks of eicosanoid metabolism and signaling at the physiological and pathological states remains to be extremally important (Serhan, 2014).

Exposure to polycyclic aromatic hydrocarbons (PAHs) usually occurs by breathing contaminated air or by eating grilled foods. As a ligand for the aryl hydrocarbon receptor (AhR), BaP and other PAHs up-regulate the expression of phase I bioactivation and phase II conjugation genes. Induction of biotransformation enzymes, including CYP1A1, CYP1B1 and epoxide hydrolase, metabolically activate BaP to different types of metabolites, including hydroxylated intermediates, epoxides and various metabolite-conjugates in cells (Kang et al., 2011; Kim et al., 2013). PAHs toxicity results from the bioactivation to the ultimate toxic epoxide compound and oxidative stress. There is strong evidence to suggest that oxidative stress is one of the most potent inductors of vascular inflammation in atherogenesis. Reactive oxygen species are known to change the redox state of exposed cells, and it is known that several inflammatory genes and the related transcription factors are regulated through redox-sensitive mechanisms (Ji et al., 2013).

Studies over the last four decades have shown that diet can modulate the response of organisms to drug absorption, distribution, metabolism, and excretion. An important mechanism for the health-promoting effects of n-3 polyunsaturated fatty acids (PUFAs) is the suppression of the pro-inflammatory metabolism of arachidonic acid (AA). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and their derivatives act as anti-inflammatory and pro-resolving compounds, inhibit the formation of AA derivatives *via* multiple mechanisms (Davidson et al., 2012; Colas et al., 2014; Spite et al., 2014). Endogenous mediators of inflammation and the mechanisms involved in regulating this process and its resolution are of wide interest (Spite et al., 2014; Gdula-Argasińska et al., 2015a,b,c).

DHA is converted through a series of enzymatic oxygenations to protectins and D series resolvins. Resolvin D2 (7(S),16(R),17(S)-trihydroxy-DHA, RvD2) is produced physiologically from the sequential oxygenation of DHA by 15- and 5-lipoxygenase and is a product of transcellular biosynthesis with human leukocytes and endothelial cells (Sun et al., 2007; Serhan and Petasis, 2011; Serhan, 2014). Resolvins D are specialized pro-resolving mediators showing dose-dependent actions on inflammatory signaling. Resolvins also specifically interact with human phagocytes via the ALX and GPR32 receptors. The mechanisms through which resolvins exert their biological actions involve down-regulation of NF-κB and AP-1 activity as well as PPARs pathways (Chatterjee et al., 2014).

The aim of this study was to evaluate the anti-inflammatory or pro-resolving impact of RvD2 on the inflammation-related proteins in the RAW 264.7 cells exposed to PAHs.

MATERIALS AND METHODS

Reagents

Prostaglandin F3α (PGF3α), prostaglandin F2a (PGF2a), 8-iso prostaglandin F3α (8-iPGF3α), 8-iso prostaglandin F2α (8-iPGF2α), 5-isoprostane F2α (5-iPF2α), prostaglandin F2α-d_9 (PGF2α -d9) and 8-iso prostaglandin F2α-d_4 (8iPGF2α -d4), RvD2 were obtained from Cayman Chemical Company (Michigan, USA). LC-MS grade methanol, ethanol, electrospray calibrate solution, butylated hydroxytoluene (BHT), PAHs, potassium hydroxide, hydrogen peroxide, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Analytical grade formic acid, chloroform, n-hexane were supplied by Merck (Darmstadt, Germany). Water (18.2 MΩcm, TOC< 5ppm) was ultrapurified and filtered through a Milli-Q Plus system (Millipore, Bedford, USA).

Cultured cells

Mus musculus murine macrophages (RAW 264.7, TIB-71, ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 1% antibiotic solution (100 IU/ml penicillin, 0.1 µg/ml streptomycin) (ATTC). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and were finally seeded into a 6-well plate (Sarstedt, Germany) at a density of 5 x 10^5 cells/well in 2 ml of medium. At every step of the procedure, cell morphology was investigated by an inverted light microscope (Olympus, Japan). Cell viability during culturing was assessed with a Trypan Blue Exclusion Test. RAW 264.7 were treated with 1µmol of benzo(a) pyrene (BaP), chrysene (Chr), fluoranthene (Flu) and benzo(a)anthracene (Baa) (Sigma-Aldrich, USA) for 1h and after incubation 40nmol of resolvin D2 (Cayman Chemical) was added for 30min. PAHs were dissolved in DMSO. RvD2 was dissolved in ethanol. Control cultures received the same concentration of ethanol (the final content did not exceed 0.12% v/v) and DMSO (the final content did not exceed 0.05% v/v) as the experimental cells. After incubation, media and the cells after scrapping were collected.

ApoTox-Glo Triplex Assay

After treatment of the cells, the ApoTox-Glo Assay was used to assess cell viability, the potential cytotoxicity of RvD1, BaP, Chr, Flu and Baa, and cell apoptosis. The assay was performed as per manufacturer instuctions (Promega, USA) as described previously (Gdula-Argasińska et al., 2015a).

Isolation of isoprostanes

Isolation of isoPs was done according to the method proposed by Milne et al. (2006). Prior to extraction, 10 µl of 0.01% BHT was added to each sample to prevent further oxidation. Then, PGF2α-d9 and 8iPGF2α-d4 (20 ng/µl) were added to the samples as an internal standard. Solid phase extraction (SPE) was done using Bond Elute Plexa cartridges (Agilent Technologies). Next, SPE samples were reconstituted in 200 µl of methanol containing 5% formic acid prior to UHPLC/MS-TOF analysis.

UHPLC/MS-TOF conditions

Identification and determination of isoprostanes were performed using an UltiMate 3000 RS liquid chromatography system (Dionex, USA) coupled to a mass spectrometer with a time-of-flight mass analyzer (MicroTOF-Q II, Bruker, Germany). Separation of the studied prostaglandins was carried out on a Synergi 4µ Hydro-RP 80A column (150 x 2,0 mm I.D, Phenomenex, USA) at 40°C. The mobile phase was prepared by mixing methanol and 0.01% formic acid according to a gradient program. The $[M-H]^-$ masses with the defined retention time (Rt) for the studied IsoPs were: 8-iPGF3α m/z=351.217±0.005 (Rt=16.3 min), PGF3α m/z= 351.217±0.005 (Rt=17.1 min), 8-iPGF2α m/z=353.232±0.005 (Rt=17.2 min), 5-iPF2α m/z=353.232±0.005 (Rt=17.4 min), PGF2α-d9 m/z=362.289±0.005 (Rt=17.2 min), 8iPGF2α-d4 m/z=357.257±0.005 (Rt=18.0 min). The details of the method were described previously (Gdula-Argasińska et al., 2013).

Western blot for quantity of COX – 2, cPGES and FP receptor

Cell lysates were prepared using M-PER mammalian protein extraction reagent (Thermo Scientific, USA) with protease inhibitor cocktail set III (Calbiochem, Merck, Germany). Protein concentrations were determined using the Bradford reaction. Aliquots (50μg) will be solubilised in a Laemmli buffer with 2% mercaptoethanol (BioRad) and subjected to 10% SDS-polyacrylamide gel electrophoresis as described below (Gdula-Argasińska et al., 2015b). We used primary antibodies: anti-cyclooxygenase-2 (COX-2), anti-prostaglandin E synthase, anti-GAPDH diluted 1:1000 in Signal+ for Western Blot (GeneTex) and FP receptor (Cayman Chemical) diluted 1:200 in Signal+, and secondary antibody Easy Blot anti rabbit IgG (HRP) (GeneTex, 1:2000). Proteins were detected using the Western blotting detection kit Clarity Western ECL Luminol Substrate (Bio-Rad, USA). The integrated optical density of the bands were quantified using Chemi Doc Camera with Image Lab software (BioRad).

Statistics

All the data are presented as means ±SD of at least six independent experiments. Comparisons between study groups were determined by one-way ANOVA followed by the Tukey's *post-hoc* test. Calculations were performed using Statistica 10 (StatSoft, USA) software, and statistical significance was established as $P \leq 0.05$.

RESULTS

No cytotoxic effects were observed in the RAW 264.7 cells treated with BaP, Chr, Flu, Baa for 2h and RvD2 for 30 min. Caspase -3/7 activity was mildly increased in the cells treated with BaP. Cell viability varied from 100% to 95% after incubation with BaP for 2h. There were no apoptotic cells after treatment with the compounds.

Isoprostane content

In control samples as well as the cells incubated with RvD2 there were no isoprostanes (Tab. 1). In RAW 264.7 cells treated with PAHs we detected all of the analyzed eicosanoids. The level of PGF2α varied from 0.8 to 1.3 ng/ml. The highest level of 8-isoPGF2α was found in the macrophages after the exposure to BaP. Also higher concentrations of PGF3α, 8-isoPGF3α were noted. In the Chr and Flu samples we identified PGF3α and 8-iso PGF3α. The levels of those compounds were lower compared with the BaP samples. RvD2 incubation resulted in a statistically decreased concentration of all isoprostanes in cells after the exposure to BaP (Tab. 1).

TABLE 1. The eicosanoid content [ng/ml] of RAW 264.7 cells after the exposure to PAHs and incubation with RvD2. Means ±SD, n=6. Asterisks denote statistically significant differences, $P<0.05$.

	8iPGF3α	**PGF3α**	**8iPGF2α**	**PGF2α**	**5iPF2α**
Control	–	–	–	–	–
RvD2	–	–	–	–	–
BaP	1.3 ± 0.5*	0.6 ± 0.0*	2.5 ± 0.4*	1.3 ± 0.2*	0.5 ± 0.1
Chr	–	0.4 ± 0.1	0.8 ± 0.2	–	–
Flu	–	0.3 ± 0.0	–	–	–
Baa	–	0.6 ± 0.1*		0.8 ± 0.2	–
RvD2+BaP	0.5 ± 0.2**	0.2 ± 0.0**	0.6 ± 0.2**	0.2 ± 0.0**	–
RvD2+Chr	–	0.3 ± 0.0	–	–	–
RvD2+Flu	–	–	–	–	–
RvD2+Baa	–	0.3 ± 0.1**	–	–	–

COX-2, cPGES and FP-receptor expression

The highest amount of COX-2 compared with the control was observed in the RAW 264.7 cells incubated with BaP. Higher expression of this protein was also observed in all the cells supplemented with other PAHs (P=0.01). The lowest expression of this protein was observed in the cells treated with PAHs and incubated with RvD2 (P=0.01) (Fig. 1A). The highest expression of cPGES pro-

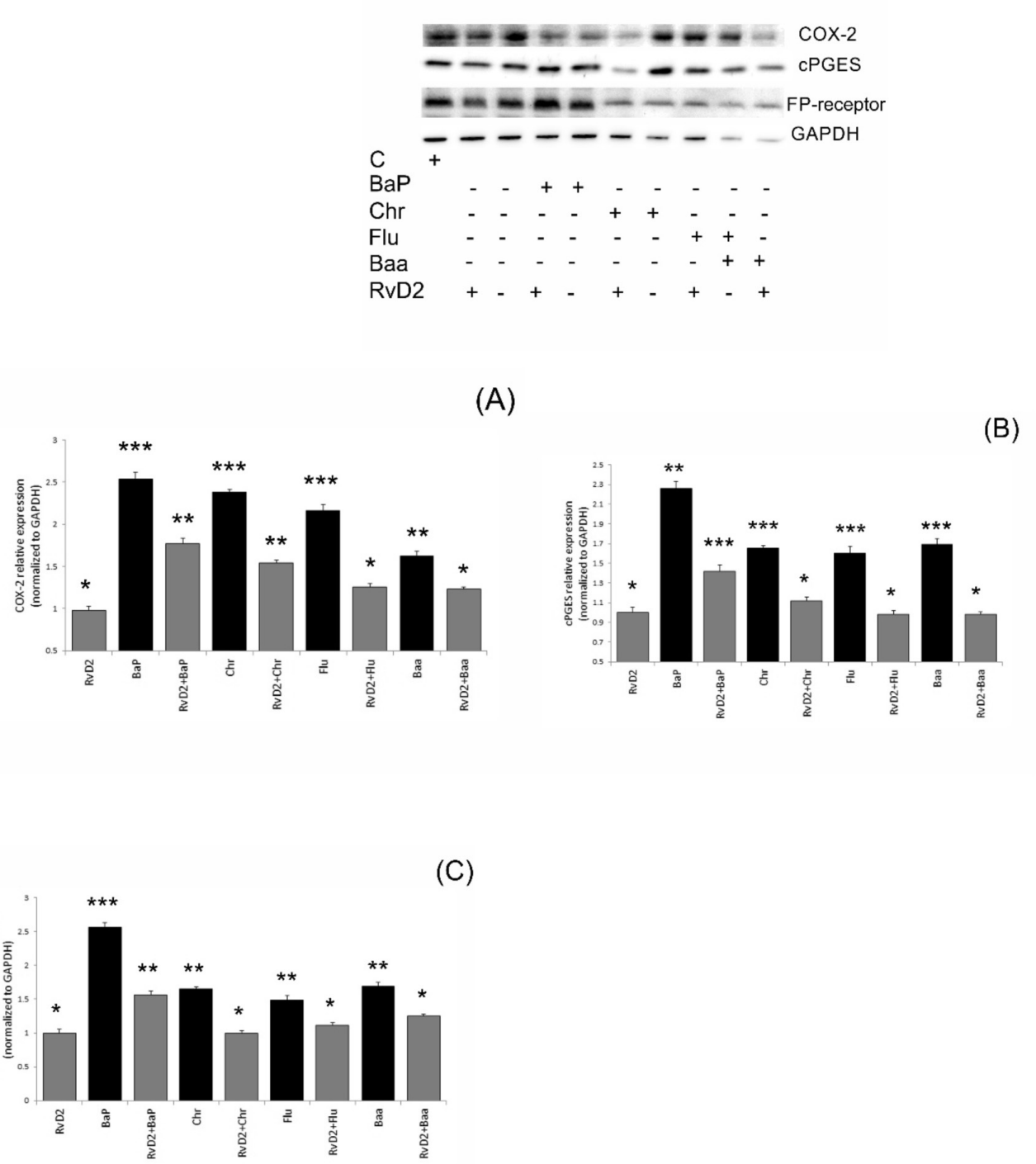

Fig. 1. Relative expression of COX-2 (A), cPGES (B) and FP-receptor (C) in RAW 264.7 cells treated with PAHs and incubated with RvD2. Asterisks denotes statistically significant differences at P<0.05.

tein was observed in BaP-treated cells (P=0.02). After the exposure to PAHs and incubation with RvD2 the expression of this protein was statistically significantly decreased (P=0.01) (Fig. 1B). In the samples incubated with PAHs and RvD2 the level of the FP-receptor was statistically lower compared with the groups exposed to PAHs only (P=0.01) (Fig. 1C).

DICUSSION

PAHs may exert negative effects on the human organism by inducing the oxidative stress. Lipid peroxidation has two major impacts on the cell. It changes the properties of the cellular membranes, affects their structure and the activity of membrane-bound proteins, and it causes the formation of other reactive intermediates that propagate oxidative stress (Kang et al., 2010; Kim et al., 2013; Ji et al., 2013). Among the markers of lipid peroxidation, isoprostanes are considered to be the most reliable. They are formed by a free-radical attack on arachidonic acid localized in cellular membranes. Currently, 8-iso prostaglandin F2α is the best characterized and the most often studied isoprostane (Milne et al., 2006; Brooks et al., 2008).

The results of our investigation have shown that the exposure to PAHs and incubation with RvD2 did not inhibit the growth and proliferation of RAW 264.7 cells. In the control cell samples and in the cells supplemented with RvD2, isoprostanes were not found. In the RAW 264.7 cells supplemented with RvD2 with the addition of BaP, active lipid derivatives were identified. We detected lipid derivatives from EPA (prostaglandin PGF3α and isoprostane 8-iPGF3α) as well as from arachidonic acid (8-iPGF2α and 5-iPF2α-VI). These findings strongly suggest that benzo(a)pyrene acts *via* cyclooxygenase pathway (formation of PGF3α) and propagates oxidative stress by lipid oxidation. This observation is similar to the results of our previous study (Gdula-Argasińska et al., 2013). Oxidative stress caused by lipid peroxidation through free radicals is believed to be one of the key factors underlying several acute and chronic diseases which cause high morbidity and mortality, and oxidative stress has been implicated in the ageing process.

The dietary intake of fatty acids affects production of eicosanoids which are potent immune mediators being mainly synthesized from eicosapentaenoic acid (EPA) and arachidonic acid (AA). Docosahexaenoic acid (DHA) and EPA are n-3 PUFAs mainly derived from fish oils that competitively inhibit n-6 PUFA arachidonic acid metabolism, thus reducing generation of the inflammatory leukotrienes and prostaglandins as well as the production of cytokines from inflammatory cells (Gao et al., 2006; Kitz et al., 2010; Serhan, 2014).

While prostaglandins are produced as a result of cyclooxygenase enzyme activity, isoprostanes are generally thought to be formed non-enzymatically by free radical-mediated peroxidation of arachidonic acid and other unsaturated fatty acids. Separate evidence suggests that cyclooxygenase activity may also contribute to isoprostane production in selected tissues. Due to the potential role of isoprostanes in the pathogenesis of a disease, their cellular signalling pathways and biological effects have been under investigation (Davidson et al., 2012; Gdula-Argasińska et al., 2013, 2015a,b,c).

In the cells treated with PAHs, and then incubated with RvD2, a decrease in COX-2, cPGES and FP-receptor expression was observed. The anti-inflammatory properties of n-3 FAs and their pro-resolving properties were observed in our previous studies. In the A549 cells supplemented with EPA or DHA and activated with lipopolysaccharide (LPS), maresin and protectin D1 and D2 were detected (Gdula-Argasińska et al., 2015b). In another study, human hepatoma HepG2 cells were used to assess the effect of supplementation with EPA and treatment with BaP. We observed the presence of lipid derivatives from EPA – Prostaglandin F3α (PGF3α), 8-iso Prostaglandin F3α (8-isoPGF3α) as well as AA derivatives. We demonstrated that EPA has an anti-oxidative stress effect under benzo(a)pyrene exposure. Our findings strongly suggest that EPA plays a role in the enhancement of anti-oxidant defence (Gdula-Argasińska et al., 2013). In the study of Gdula-Argasińska et al., (2015c) it has been shown that supplementation of human epithelial cells A549 with EPA and DHA results in up- (AhR and PLA2G4A) and down-regulation (PTGS2) of genes and higher phospholipase A2 activity.

The results from the study of Merched et al. (2008) indicate that 12/15-lipoxygenase expression protects mice against atherosclerosis *via* its role in the local biosynthesis of lipid mediators, including lipoxin A4, resolvin D1, and protectin D1. These mediators exert potent agonist actions on macrophages and vascular endothelial

cells that can control the magnitude of the local inflammatory response (Merched et al., 2008; Serhan, 2014). Findings from the study of Lee et al. (2013) indicate that RvD1 expedites resolution of inflammation in macrophages through induction of efferocytosis by p50/p50-homodimer-mediated repression of TNF-a production.

Altogether, our findings provide a novel mechanism underlying the detrimental effects of PAHs and RvD2 on macrophages which might play a critical role in inflammatory disease states. Our results suggest that RvD2 plays a role in enhancement of the anti-oxidant, anti-inflammatory and pro-resolving activities and has a high therapeutic value and therefore should be one of the therapeutic approaches due to its dynamically modulatory properties.

ACKNOWLEDGEMENTS

Supported by the grant from National Science Centre, Poland, No:DEC-2011/01/B/NZ7/00038 given to Joanna Gdula-Argasińska.

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