Volume 279, number 1, 110–114 FEBS 09387 February 1991

ADONIS 001457939100069X

The oxygenation of cholesterol esters by the reticulocyte lipoxygenase

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Received 3 December 1990

The arachidonate 15-lipoxygenase from rabbit reticulocytes oxygenates cholesterol esters containing polyenoic fatty acids. Cholesterol esterified with saturated fatty acids is not oxygenated. The structures of the oxygenation products formed from various cholesterol esters have been identified by high pressure liquid chromatography, UV-spectroscopy and gas chromatography/mass spectroscopy. Oxygenated cholesterol esters have been detected in atherosclerotic plaques of human aortas.

Lipoxygenase; Cholesterol ester; Atherosclerosis

1. INTRODUCTION

The lipoxygenase of rabbit reticulocytes is able to oxygenate not only free polyenoic fatty acids but also more complex substrates, such as phospholipids [1] and biological membranes [2]. Some years ago, Nekrasov and coworkers [3] investigated the interaction of this enzyme with cholesteryl arachidonate and concluded from the spectral changes of the incubation mixture that, in contrast to the soybean enzyme, the reticulocyte lipoxygenase may oxygenate cholesterol esters.

Cholesterol esters are important constituents of human lipoproteins, particularly of LDL. In the past few years it became generally accepted that oxidatively modified LDL play an important role in the early events of the pathogenesis of atherosclerosis [4,5]. The mechanism of the reactions leading to the oxidative modifications of the lipoproteins are not completely understood. There is, however, a substantial body of experimental evidence which strongly suggests that autoxidation processes [6] may lead to oxidative modification lipoproteins. On the other hand, Steinberg and coworkers found that the arachidonate 15-lipoxygenase of the endothelial cells may also be involved in these processes [7]. More recently, McNally et al. showed that human monocytes are able to oxidize LDL via the lipoxygenase pathway [8]. The recent reports of the possible involvement of lipoxygenases in the oxidative modification of lipoproteins prompted us to investigate the interaction of a mammalian 15-lipoxygenase with cholesterol esters which are important constituents of lipoproteins. The data presented indicate that cholesterol esters can be oxygenated by lipoxygenases and that the product pattern formed was very similar to that observed for the oxygenation of the free fatty acids.

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Abbreviations: SP-HPLC, normal phase high-pressure liquid chromatography; RP-HPLC, reverse phase high-pressure liquid chromatography; 13S-HODE, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; 15S-HETE, 15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; 9-HODE, 9-hydroxy-10,12-octadecadienoic acid; 13-HOTE, 13-hydroxy-9,11,15-octadecatrienoic acid; GC/MS, gas chromatography/mass spectrometry; MSTFA, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide; DNBPG, dinitrobenzoyl phenylglycine; LDL, low density lipoproteins

2. MATERIALS AND METHODS

2.1. *Chemicals*

The chemicals used were from the following sources: cholesteryl linoleate, cholesteryl arachidonate, cholesteryl linolenate and sodium cholate from Serva (FRG); sodium borohydride and silica gel (0.05–0.2 mm particle size) from Merck (Germany). All solvents used were of analytical grade and distilled prior to use.

2.2. *Oxygraphic assay*

1 mg of cholesterol esters was suspended in 10 ml of 0.1 M phosphate buffer, pH 7.4, containing 0.1% sodium cholate and sonicated with a Branson B-12 sonifier for 1 min at 100 W. The turbid suspension was incubated with the reticulocyte lipoxygenase (40 nkat/ml linoleate oxygenase activity* for 10 min at room temperature in a 2 ml oxygraphic assay chamber equipped with a Clark-type oxygen electrode. The oxygraphic scale was calibrated by oxidation of known amounts of NADH by beef heart submitochondrial particles [2].

2.3. *Preparations*

The reticulocyte lipoxygenase was purified as described in [9]. Peak fractions of the isoelectric focusing (pure enzyme) or the ammonium

*1 nkat is defined as substrate turnover of 1 nmol/s

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sulfate precipitate (crude enzyme preparation) was used for the experiments.

For the preparation of human plasma cholesterol esters, 100 ml of blood from a healthy volunteer was withdrawn. The plasma lipids were extracted [10] and the cholesterol esters were separated from the other plasma lipids by open bed silica column chromatography [11].

For elucidation of their chemical structure the oxygenated cholesterol esters were prepared as follows. After a 10 min incubation of the different substrates with the lipoxygenase the reaction was stopped by the addition of sodium borohydride and the lipophilic products were extracted [10]. The solvent was evaporated, the residue was dissolved in chloroform and aliquots were injected to HPLC or subjected to alkaline hydrolysis (30 min incubation at 60°C in a mixture of methanol/6 N KOH (5:1 v/v) under argon).

The thoracic aorta of 5 men (45–65 years old) who had suffered from chronic ischemic heart disease and who died from actute heart failure was removed during autopsy. From each aorta one region with signs of atherosclerotic changes and 'normal' looking aortic tissue were prepared immediately after removal of the organs, the wet weight was determined and the aorta pieces were shock-frozen on dry-ice and stored on dry-ice under a CO_2 atmosphere. After thawing, the total tissue lipids were extracted [10] by homogenization of 1 g of aortic tissue in 6.25 ml of a mixture of methanol/chloroform/water (2.5:2.5:1.25, v/v) with an Ultra-Turax homogenizer. After phase separation, the lower phase containing the lipids was recovered and the solvent was removed under vacuum. The residue was redissolved in 1 ml of chloroform and the samples were stored under a argon atmosphere at −20°C.

Authentic standards of the hydroxy polyenoic fatty acids were prepared by vitamin E controlled autoxidation [12]. Methylation of free fatty acids, catalytic hydrogenation of double bond and silylation of OH-groups were carried out as described in [2].

2.4. *Analytics*

HPLC analysis was performed on a DuPont instrument coupled with a Hewlett Packard diode array detector 1040 A. The separation of the oxygenated and non-oxygenated cholesterol esters [13] was carried out on a Nucleosil column (250 mm × 4 mm, 5 μm particle size; Macherey/Nagel [FRG], KS-system) with a solvent system acetonitrile/2-propanol (75:25; v/v); flow rate of 1 ml/min, separation temperature 45°C. The absorbances at 235 nm and 210 nm were recorded simultaneously. RP-HPLC was performed at room temperature on a Zorbax ODS column (250 mm × 4.6 mm, 5 μm particle size) with a solvent system methanol/water/acetic acid (82:18:0.1 v/v); flow rate 1 ml/min. SP-HPLC of the oxygenated polyenoic fatty acids was carried out on a Zorbax-SIL column (250 mm × 4.6 mm, 5 μm particle size) with the solvent system *n*-hexane/2-propanol/acetic acid (100:2:0.1; v/v); flow rate 1 ml/min. For chiral phase HPLC a Bakerbond (R)-DNBPG column (250 mm × 4.6 mm, 5 μm particle size, ionically linked chiral phase; Baker Chem., USA) was used. The solvent system was *n*-hexane/2-propanol (100:0.25; v/v); flow rate 1 ml/min [14].

GC/MS of the silylated hydroxy fatty acid methyl esters was carried out on a Shimadzu GC/MS-system (QP 2000) equipped with a RSL-150 column (polydimethylsiloxane, 30 m × 0.32 mm, coating thickness 0.25 μm). Temperature program: 2 min at 180°C, then at a rate of 5°C/min to 250°C, injector temperature 275°C, ion source temperature 180°C, electron energy 70 eV.

3. RESULTS

3.1. *Cholesterol esters are substrates for the reticulocyte lipoxygenase*

The incubation of the pure reticulocyte lipoxygenase with different cholesterol esters is accompanied by an oxygen uptake indicating that this enzyme is able to oxygenate cholesterol esters containing polyenoic fatty acids. In contrast, cholesterol esters containing saturated fatty acids (cholesteryl myristate) were not oxygenated. Among the synthetic cholesterol esters cholesteryl linoleate turned out to be the best substrate (Fig. 1). Similarly, free linoleic acid was found to be a better substrate than linolenic acid and arachidonic acid (data not shown). The cholesterol esters prepared from the human plasma lipids were also well oxygenated. It should be stressed that the oxygenation rate of the free polyenoic fatty acids is almost three orders of magnitude higher than that of the corresponding cholesterol esters.

3.2. *Isolation of oxygenated cholesterol esters*

In order to find out which products are formed during the oxygenation of the different cholesterol esters the lipophilic products were extracted after a 10 min incubation of the enzyme with the different substrates. The hydroperoxy derivatives proposed to be formed were reduced with sodium borohydride and the resulting hydroxy compounds were analyzed by HPLC. In Fig. 2A a representative chromatogram of the non-oxygenated cholesterol esters isolated from human plasma is shown. Recording the chromatogram at 210 nm allows the detection of the different cholesterol esters occurring in the human plasma. The various cholesterol esters have been identified by coinjections with authentic standards and by GC/MS of their cholesterol and their fatty acid component obtained after alkaline hydrolysis. Recording the chromatogram at 235 nm (Fig. 2B) indicated that only small amounts of conjugated dienes were present. After lipoxygenase treatment the chromatogram at 210 nm did look very similar to that of the non-oxygenated sample (not shown), but at 235 nm (Fig. 2C) an additional compound with a retention time of 10.9 min was detected. This compound was characterized by a typical con-

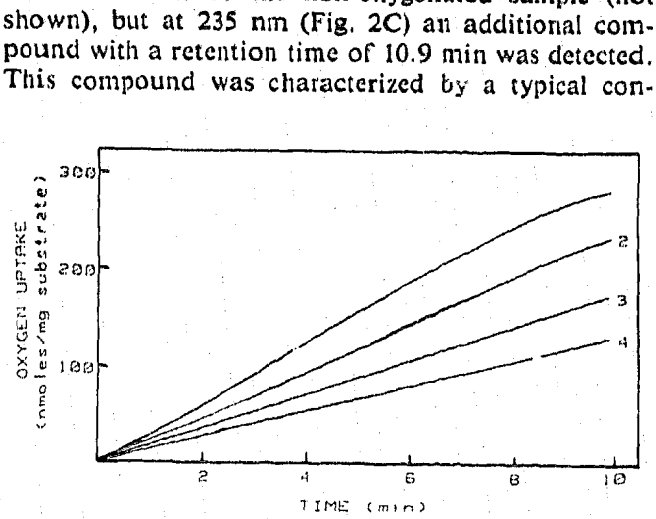

Fig. 1. Oxygenation of different cholesterol esters by the reticulocyte lipoxygenase. 200 μg of the cholesterol esters were suspended in 2 ml of 0.1 M phosphate buffer, pH 7.4, containing 0.2% sodium cholate. The reaction was started by the addition of the reticulocyte lipoxygenase. (1) Cholesterol esters prepared from human blood plasma; (2) cholesteryl linoleate; (3) cholesteryl arachidonate; (4) cholesteryl linolenate.

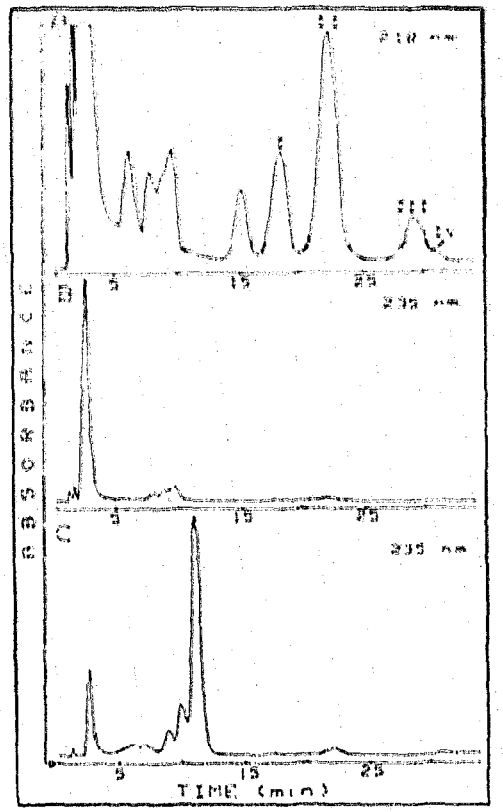

Fig. 2. SP-HPLC of the cholesterol esters from human blood plasma before and after lipoxygenase treatment. The cholesterol esters prepared from human blood plasma (100 μg/ml) were incubated with reticulocyte lipoxygenase as described in Materials and Methods. After borohydride reduction the lipids were extracted and analyzed by SP-HPLC with the solvent system acetonitril/2-propanol (75:25, v/v) and a flow rate of 1 ml/min. The absorbances at 210 and 235 nm were recorded simultaneously. (A) Before lipoxygenase treatment, detection at 210 nm. (B) Before lipoxygenase treatment, detection at 235 nm. (C) After lipoxygenase treatment, detection at 235 nm. I, cholesteryl arachidonate; II, cholesteryl linoleate; III, cholesteryl oleate; IV, cholesteryl palmitate.

jugated diene chromophore with an absorbance maximum at 234 nm. An almost identical chromatogram was obtained if the oxygenated cholesterol esters were not reduced with borohydride (not shown) indicating that hydroxy and hydroperoxy derivatives of the cholesterol esters have similar retention times under our chromatographic conditions. Furthermore, we did not detect any differences in the product pattern if the oxygenation was carried out with the pure enzyme (peak fraction of isoelectric focussing) or an crude enzyme preparation (ammonium sulfate precipitate). Oxygenation of the commercially available cholesterol esters did also lead to the formation of products containing a conjugated diene chromophore. The retention times of the products formed from the different substrates were somewhat different: 10.8 for the oxygenated cholesteryl linoleate, 9.8 for the oxygenated cholesteryl linolenate and 8.9 for the oxygenated cholesteryl arachidonate.

3.3. *Structure elucidation of the oxygenation products*

To obtain more detailed information on the structure of the oxygenation products, the compounds absorbing at 235 nm were prepared by SP-HPLC and hydrolyzed under alkaline conditions. The conjugated dienes were prepared by RP-HPLC and further analyzed by SP-HPLC. In Fig. 3 a representative chromatogram of lipoxygenase-treated human plasma cholesterol esters is shown. It can be seen that 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE)[b] was the main product. Other hydroxy-octadecadienoates (9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE) and the two all E-isomers) and 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE) could also be detected. Chiral phase HPLC indicated that for both 13-HODE and 15-HETE the S-isomer was predominant whereas the other products turned out to be racemic mixtures (Table I). Similar results were obtained with the commercially available cholesterol esters. In Table I the results of the structure elucidation of the products formed during the oxygenation of different substrates by the reticulocyte lipoxygenase are summarized.

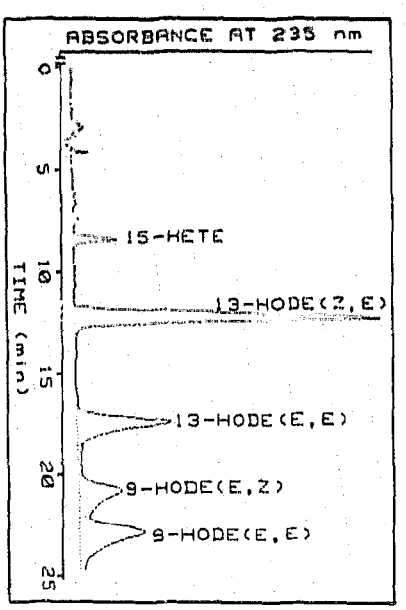

Fig. 3. SP-HPLC of the fatty acid component of cholesterol esters from human blood plasma after lipoxygenase treatment. The cholesterol esters obtained from human blood plasma were oxygenated with the reticulocyte lipoxygenase (pure enzyme) as described in Materials and Methods. After borohydride reduction, lipid extraction and alkaline hydrolysis the oxygenated polyenoic fatty acids were prepared by RP-HPLC and further analyzed by SP-HPLC with the solvent system: *n*-hexane/2-propanol/acetic acid (100:2:0.1, v/v).

[b]The chemical structure of all products has been identified by cochromatography with authentic standards, by UV-spectroscopy and GC/MS of the native trimethylsilyl ethers and their hydrogenated derivatives

TABLE I

Product composition formed during the oxygenation of various cholesterol esters by the reticulocyte lipoxygenase

Substrate	Products			
Cholesteryl linoleate	13-HODE (Z,E) 71% (82:18)	13-HODE (E,E) 4%	9-HODE (E,Z) 13%	9-HODE (E,E) 12%
Cholesteryl arachidonate	15-HETE 65% (64:36)	12-HETE 16%	11-HETE 19%	
Cholesteryl linolenate	13-HOTE (82%) (89:11)	12-HOTE (2%)	16-HOTE (11%)	9-HOTE (5%)
Human plasma cholesterol ester	13-HODE (Z,E) 43% (80:20)	13-HODE (E,E) 13% (50:50)	9-HODE (Z,E) 19% (55:45)	9-HODE (E,E) 22% (53:47)
	15-HETE 3% (87:13)			

The cholesterol esters were oxygenated with the reticulocyte lipoxygenase as described in Materials and Methods. After borohydride reduction, lipid extraction and alkaline hydrolysis the resulting hydroxy fatty acids were prepared by RP-HPLC and analyzed by SP-HPLC. In parenthesis the S/R-ratio (determined by chiral phase HPLC) is given.

3.4. *Detection of oxygenated cholesterol esters in atherosclerotic plaques of human aorta*

We examined the lipid extracts of atherosclerotic lesions and 'normal' looking regions of 5 different aortas. In Fig. 4 a representative HPLC analysis of a lipid extract of an atherosclerotic plaque is shown. It can be seen that the pattern of the non-oxygenated cholesterol esters (lower trace) is very similar to that detected in the human plasma. In addition, large amounts of free cholesterol were found. Recording the chromatogram at 235 nm (upper trace) oxygenated cholesterol esters were detected. The amount of these compounds varied between 17 and 55 mg/g wet weight for the 5 samples.

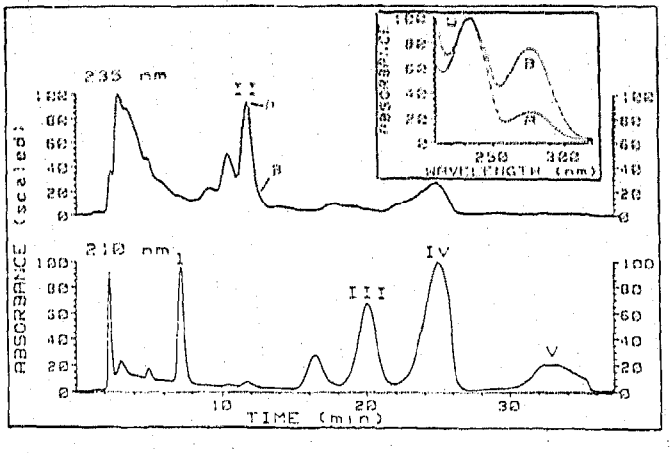

Fig. 4. SP-HPLC of the cholesterol esters isolated from atherosclerotic lesions of human aorta. The total lipids were extracted from atherosclerotic plaques of a human aorta as described in Materials and Methods. The cholesterol esters were analyzed by SP-HPLC with the solvent system acetonitril/isopropanol (75:25, v/v) and a flow rate of 1 ml/min. The absorbance at 210 nm and 235 nm were recorded simultaneously. I, free cholesterol; II, oxygenated cholesterol esters; III, cholesteryl arachidonate; IV, cholesteryl linoleate; V, cholesteryl oleate and cholesteryl palmitate. Inset: UV-spectrum recorded at the times indicated.

Calculation of the oxygenated/non-oxygenated cholesterol ester ratio indicated that 12.4–21% (in the 5 different samples) of the cholesteryl linoleate was present as oxygenated derivatives. In order to exclude that the oxygenated cholesterol esters were formed during the work-up procedure, radioactively labelled methyl linoleate was added to the homogenization mixture. In HPLC analysis no oxygenated methyl linoleate derivatives were detected. In 'healthy' looking regions of the same aortas much smaller amounts of oxygenated cholesterol esters were detected (ranging from 0.3 to 4.5 mg/g wet weigth). In these samples the oxygenated/non-oxygenated cholesteryl linoleate ratio ranged from 5.8% to 9.5%. The differences in the oxygenated/non-oxygenated cholesterol ester ratios in 'healthy' looking regions and atherosclerotic lesions of each aorta were highly significant ($P<0.001$) as indicated by the paired t-test.

Continuous recording of UV-spectra across the peak (peak purity search) indicated that the peak was not homogeneous but consisted at least of two different compounds with different spectral properties (inset to Fig. 4). Alkaline hydrolysis of the compounds absorbing at 235 nm and SP-HPLC of the resulting free fatty acid derivatives indicated a complex mixture of oxygenated linoleic acid derivatives.

4. DISCUSSION

The data presented here indicate that the lipoxygenase from rabbit reticulocytes, a mammalian arachidonate 15-lipoxygenase, is able to oxygenate cholesterol esters which contain polyenoic fatty acids. These findings might be of importance for the pathogenesis of the early events of atherosclerosis. It has been suggested before that the oxygenation of low density lipoproteins by the endothelial cell or monocyte lipoxygenase may lead to oxidative modifications of the

lipoproteins [7,8]. These modified lipoproteins but not the non-oxygenated ones can bind to the scavenger receptor of monocyte-derived macrophages [4,14] and are subsequently taken up by the cells. These processes lead to the formation of foam cells what is commonly regarded as one of the early events in the pathogenesis of atherosclerosis [15]. Since cholesterol esters are an important part of the lipid core of LDL, they might serve as substrate for the cellular lipoxygenase in vivo. Recently, we found that LDL cholesterol esters are oxygenated during the in vitro interaction of the reticulocyte lipoxygenase with low density lipoproteins without the preceding action of a phospholipase (unpublished data).

As compared with free fatty acids, the oxygenation rate of cholesterol esters is about three orders of magnitude lower. This finding is not surprising since other complex lipoxygenase substrates such as biological membranes [2] or lipoproteins (unpublished data) are also oxygenated with a low rate. It is proposed that the availability of the polyenoic fatty acid residues for the enzyme is sterically hindered in the complex substrates. In the case of the cholesterol esters, the low water solubility may also contribute to the low oxygenation rate.

The product specificity of the oxygenation of cholesterol esters is very similar to that of other substrates [1,2,17]. With the human plasma cholesterol esters, however, the product specificity was not very high since substantial amounts of racemic side products were formed. Similar results have been reported for the oxygenation of biomembranes by the pure reticulocyte lipoxygenase [2]. The product specificity of the lipoxygenase reaction appears not to be an absolute enzyme property but depends on the reaction conditions, such as substrate concentration or reaction temperature [2]. Therefore, an unspecific product pattern does not necessarily exclude the lipoxygenase origin of oxygenated lipids.

The detection of oxygenated cholesterol esters in atherosclerotic plaques of human aorta confirms earlier findings by Harland et al. who isolated hydroperoxides of cholesteryl linoleate from the lipids of advanced atherosclerotic plaques [18]. Our results suggest that the degree of oxidative modification of the aorta lipids (quantified by the oxygenated/non-oxygenated cholesterol ester ratio) appears to correlate with the stage of the atherosclerotic lesion. In fibrous plaques, a high oxygenated/non-oxygenated cholesterol ester ratio was found. In 'normal' looking aortic tissue this ratio was significantly lower. In the fatty streaks which are recognized as early atherosclerotic lesions this ratio was higher than in the 'normal' looking aorta but lower than in the plaques (data not shown). The mechanism of formation of these products in vivo remains to be clarified. It cannot be completely excluded that a small part of the oxygenated cholesterol esters are formed in situ during the time between death and autopsy. After removal of the aortas autoxidation processes were largely inhibited by the storage conditions and the work-up protocol (see Materials and Methods). According to our experiences lipid peroxidation in animal tissue does not take place if the tissue is handled according to our protocol. Furthermore, autoxidations during homogenization, lipid extraction and extract storage can be excluded as indicated by the control experiments with radioactively labelled methyl linoleate. Therefore it is concluded that the oxygenated cholesterol esters are formed in vivo and that their formation may be involved in the pathogenesis of atherosclerosis.

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