RESEARCH COMMUNICATION

Inhibition of poly(ADP-ribose) formation by 4-hydroxynonenal in primary cultures of rabbit synovial fibroblasts

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The formation of poly(ADP-ribose) in primary cultures of rabbit synovial fibroblasts after treatment with active oxygen released by xanthine/xanthine oxidase is inhibited by addition of 1 and 10 μ M 4-hydroxy-2,3-*trans*-nonenal (HNE). The endogenous formation of HNE by the xanthine/xanthine oxidase system is not responsible for the inhibitory effect of the aldehyde, owing to the low accumulation rate of the lipid peroxidation product in

the system used. HNE is able to inhibit the isolated nuclear enzyme ADP-ribosyltransferase, as shown by an *in vitro* assay with an K_i of 4 μ mol/litre. Therefore the molecular basis of HNE-mediated effects on cell proliferation, differentiation and transformation might be due to the inhibitory effect of poly(ADP-ribosylation).

INTRODUCTION

Lipid peroxidation is associated with the response to oxidative stress in inflammation [1–17] and is always combined with the formation of aldehydes. 4-Hydroxy-2,3-*trans*-nonenal (4-hydroxynonenal, HNE) is a major product of lipid peroxidation formed by the degradation of ω 6 polyunsaturated fatty acids, e.g. arachidonic acid [18]. This aldehyde is formed in inflammatory tissues such as the synovial fluid of patients with rheumatoid arthritis [19]. HNE is a rather long-living metabolite compared with the primarily formed reactive oxygen species in inflammation and is therefore able to diffuse from the site of active inflammation to the surrounding tissue [20]. So HNE, which is also chemotactic, is considered to be a messenger of inflammation. HNE has genotoxic, cytotoxic and mutagenic activity and produces a multitude of effects such as depletion of glutathione, disturbance of calcium homeostasis, inhibition of DNA, RNA and protein synthesis, initiation of lipid peroxidation and inhibition or activation of some specific enzymes [20]. Stimulation of phospholipase A_2 , chemotactic activity, modulation of adenylate cyclase activity and stimulation of phospholipase C have previously been described as effects of HNE in the pathophysiologically relevant concentration range [20].

There is strong evidence that active oxygen can act as a tumour promoter and contribute to cell transformation and that poly(ADP-ribose) is involved in this process by modulating the expression of specific genes [21–23]. The nuclear enzyme ADP-ribosyltransferase catalyses the synthesis of poly(ADP-ribose) using NAD^+ as substrate. Poly(ADP-ribose)-modified nuclear proteins are also essential for DNA repair [24]. So the repair of DNA strand-breaks shows a dependence upon the rapid synthesis and degradation of poly(ADP-ribose) [25]. Ehrlich et al. previously described a considerable accumulation of poly(ADP-ribose) in rabbit synovial fibroblasts after treatment with reactive oxygen species [26] which is likely due to oxygen-radical-mediated DNA damage. Poly(ADP-ribose)-modified nuclear proteins are also involved in the regulation of the expression of specific genes [27,28], such as that coding for procollagen [29]. HNE has also been shown to enhance the transcription of the procollagen α 1-

gene and collagen synthesis in liver ito cells [18,30]. The signal pathway between HNE accumulation and regulation of procollagen-gene transcription is completely unknown.

In the present study, primary cultures of synovial fibroblasts, isolated from the joints of rabbits, were treated with xanthine/xanthine oxidase (X/XO), which mimics the release of active oxygen species from polymorphonuclear leucocytes and monocytes/macrophages during inflammation [31]. We measured the influence of a pathophysiologically relevant HNE concentration (1 and 10 μ M HNE) on rabbit synovial fibroblasts to investigate a possible link between the inflammatory release of oxygen radicals, the formation of HNE and the intracellular formation of poly(ADP-ribose), which may result in the alteration of expression of specific genes such as that coding for procollagen.

MATERIALS AND METHODS

Materials

Xanthine sodium salt was purchased from Sigma, St. Louis, MO, U.S.A., and XO was from Boehringer, Mannheim, Germany. HNE was a gift from Professor H. Esterbauer, Institut für Biochemie, Universität Graz, Graz, Austria. Other chemicals used were of analytical grade.

Preparation of synovial fibroblasts

Monolayer cultures of rabbit synovial fibroblasts were established from the knee synovium of young rabbits as described previously [32–34]. Briefly, cells were dissociated into a single-cell suspension with bacterial collagenase (ICN Biochemicals, Cleveland, OH, U.S.A.) and plated in Dulbecco's modified Eagle's medium with 10% fetal-calf serum and penicillin/streptomycin (Gibco, Paisley, Renfrewshire, Scotland, U.K.).

Treatment of synovial cells with X/XO and HNE

The monolayer cultures (5×10^6 cells) were treated with active oxygen released by 50 μ g/ml X and 0.01 unit/ml XO for 30 min

Abbreviations used: HNE, 4-hydroxy-2,3-*trans*-nonenal; DNPH, 2,4-dinitrophenylhydrazine; X/XO, xanthine/xanthine oxidase.

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in medium 199 supplemented with 8 % fetal-calf serum. In some experiments HNE was added at the same time point.

Determination of poly(ADP-ribose)

Cells were harvested after 30 min, and the content of poly(ADP-ribose) was determined according to the method of Jacobson et al. [35], avoiding cell permeabilization. Polymers of ADP-ribose were purified by affinity chromatography on a boronate resin and subsequently digested to nucleosides by incubation with snake-venom phosphodiesterase (Worthington, Freehold, NJ, U.S.A.), followed by chemical derivatization to fluorescent analogues with chloroacetaldehyde. Fluorescent nucleoside derivatives were separated by reversed-phase HPLC (Kontron, Echting, Germany), and the fluorescence of ethenoribosyladenosine was determined with a fluorescence detector (SFM 25; Kontron; excitation wavelength 235 nm; emission wavelength 425 nm).

Determination of DNA

The DNA content was quantified as described by Labarca and Paigen [36].

Determination of endogenous HNE by derivatization with 2,4-dinitrophenylhydrazine (DNPH) [37]

Cells were harvested and suspended in Medium 199 supplemented with 8 % fetal-calf serum. A 0.1 ml portion of a 12 mM 2,6-di-*t*-butyl-4-methylphenol solution in ethanol was added. After incubation of 3.5 ml suspension with 3.5 ml of DNPH solution (solution in 1 M HCl, extraction with *n*-hexane; 1.8 mM solution adjusted by UV absorption at 378 nm) for 2 h in the dark (mixing), samples were placed in an ice bath (dark) for 1 h, followed by extraction with dichloromethane and centrifugation at 900 *g* (three times). The resulting solution was evaporated to 1 ml volume in dichloromethane for TLC. TLC plates were eluted with dichloromethane leading to the separation into three zones of carbonyl compounds. The hydroxyalkenal zone was scraped off, extracted with methanol and concentrated by evaporation [37]. The dinitrophenylhydrazones of the hydroxyalkenals were determined by HPLC separation using a 5 μ M RP18 column (Macherey und Nagel, Düren, Germany; 250 mm $\times$ 4.6 mm), a methanol/water eluent (80:20, v/v), a flow rate of 0.9 ml/min and with UV detection at 378 nm.

Determination of exogenously added HNE

HNE extracted with acetonitrile/acetic acid (94:6, v/v) was determined by HPLC separation using a 5 μ m-pore-size RP 18 column (Supelco, Bellefonte, PA, U.S.A.; 150 mm $\times$ 4.6 mm int. diam.), an acetonitrile/water eluent (40:60, v/v), a flow rate of 1 ml/min and UV detection at 223 nm.

Assay for the activity of the isolated ADP-ribosyltransferase

ADP-ribosyltransferase activity was assayed by measuring the radioactivity incorporated from [*adenine*-¹⁴C]NAD⁺ into trichloroacetic acid-insoluble material. The reaction mixture contained 100 mM Tris/HCl, pH 8.0, 200 μ M [*adenine*-¹⁴C]NAD⁺ (10000 d.p.m.), 2 μ g of calf thymus DNA (sonicated ten times for 10 s), 10 mM MgCl₂, 10 mM mercaptoethanol, 1 μ g of calf thymus histone 2a (Sigma) and 1 μ g of ADP-ribosyltransferase, isolated from human placenta, in 100 μ l. The isolated ADP-ribosyltransferase was a gift of Professor M. Schweiger (Free University, Berlin, Germany). Incubation was carried out for 10 min at 25 °C. The reaction was stopped by addition of 0.8 ml of ice-cold 15 % (w/v) trichloroacetic acid and

20 μ l of 5 % BSA as carrier. The acid-insoluble material was collected on a Whatman G/C Glass microfibre filter, washed with 5 % trichloroacetic acid and 95 % ethanol and the radioactivity was counted. We used 1 mM 3-aminobenzamide as a positive control for inhibition and the HNE-free assay as negative control for no inhibition. In the case of measurement of NAD⁺ hydrolysis, the same conditions were used with the exception that no radiolabelled [*adenine*-¹⁴C]NAD⁺ was added and the NAD⁺ hydrolysis was assayed by HPLC. HPLC separation was performed on an Supelcosil 150 mm $\times$ 4 mm LC-18-S (5 m) column using an eluent consisting of 10 mM (NH₄)H₂PO₄ with 2 mM tetrabutylammonium phosphate, pH 6.8, and 5 % acetonitrile. The separation was performed at a flow rate of 1 ml/min, and the detection wavelength was 254 nm. Peak identification and quantification were performed by comparison with known standards. NAD⁺ hydrolysis was quantified by the rate of formation of nicotinamide.

RESULTS

The synovial fibroblast cultures were incubated with two different concentrations of HNE (1 and 10 μ M) and the poly(ADP-ribose) formation, stimulated by X/XO, was measured. The experiments were performed after 30 min of stimulation of synovial fibroblasts by X/XO, where the highest poly(ADP-ribose) accumulation was detected. As shown in Table 1, both concentrations of HNE inhibit the formation of poly(ADP-ribose). HNE alone leads to no accumulation of poly(ADP-ribose).

Because of the fast metabolism of HNE in eukaryotic cells, we measured the degradation of exogenously added HNE in the synovial-cell culture. The bulk of HNE is rapidly consumed within the first few seconds by binding to the media (Figure 1). The first data point represents the amount of free HNE in the cell-free culture medium after addition of 1 or 10 μ mol/litre HNE. The following data represent the consumption of HNE by the synovial cells. It does not seem possible to use HNE concentrations lower than 1 μ M because 60 % of the 1 μ M HNE concentration is rapidly consumed, most likely by reactions with SH- and amino groups in the medium. So after addition of radiolabelled HNE, 60 % of the added radioactivity can be detected in a trichloroacetic acid precipitate of the medium. Nevertheless, after addition of 1 μ M HNE a relatively large amount of the aldehyde is still available for the direct action on various enzymes, such as the ADP-ribosyltransferase.

To test whether the endogenous formation of HNE during X/XO treatment has an influence on poly(ADP-ribose) for-

Table 1 Effects of HNE on poly(ADP-ribose) synthesis in primary synovial fibroblasts

HNE, 50 μ g/ml X and 0.01 unit/ml XO were added to the supernatant of a monolayer culture of primary synovial fibroblasts. Cells were harvested and poly(ADP-ribose) was determined after 30 min of incubation. There was no influence of X or XO alone on the poly(ADP-ribose) content of synovial fibroblasts. Abbreviation: ABA, 3-aminobenzamide. The values are means $\pm$ S.D. for three independent experiments (**P* < 0.05 versus treatment with X/XO alone).

[HNE] (μ M)	X/XO	ABA	Poly(ADP-ribose) (pmol/ μ g of DNA)
0	—	—	0.09 $\pm$ 0.02*
1	—	—	0.09 $\pm$ 0.03*
10	—	—	0.09 $\pm$ 0.02*
0	+	—	4.89 $\pm$ 0.25
1	+	—	2.53 $\pm$ 0.19*
10	+	—	3.14 $\pm$ 0.17*
0	+	+	0.38 $\pm$ 0.08*

Figure 1 Degradation of micromolar concentrations of HNE in primary synovial fibroblasts

The Figure shows the degradation of 10 (■) and 1 (●) μM initial concentration of HNE in rabbit synovial fibroblasts, measured by HPLC determination in the supernatant as described in the text. The amount of free HNE at incubation time '0' is that without the share bound to the incubation medium. Results are means for four independent experiments; the S.D. value was < 10% of the HNE value.

Table 2 Endogenous HNE formation in synovial fibroblasts during X/XO treatment

Endogenous HNE was measured before, after 10 min and after 30 min of treatment with X/XO. The values represent the means $\pm$ S.E.M. for six independent experiments (* P < 0.01 versus time point 0).

Time (min)	HNE accumulation (nmol/5 $\times 10^6$ cells)	HNE concn. (nmol/litre)
0	0.03 $\pm$ 0.01	1.5 $\pm$ 0.7
10	0.21 $\pm$ 0.02*	10.5 $\pm$ 1.0*
30	0.20 $\pm$ 0.02*	10.1 $\pm$ 0.9*

mation, we measured the endogenous HNE accumulation during X/XO treatment. An accumulation of 0.20 nmol/5 $\times 10^6$ cells after 30 min of treatment with X/XO was found, as compared with 0.03 nmol/5 $\times 10^6$ cells without any treatment (basal level of HNE). These values range between 1.5 and 10.0 nmol/litre of suspension and are about two orders of magnitude lower than the inhibitory concentration of 1 $\mu\text{mol/litre}$ (Table 2).

We therefore decided to investigate the inhibitory effect of HNE on isolated ADP-ribosyltransferase in a direct *in vitro* assay. We demonstrated the inhibition of the enzyme by HNE using the above-described assay (Figure 2). HNE inhibits the activity of the ADP-ribosyltransferase *in vitro* with a K_i of 4 $\mu\text{mol/litre}$ (estimated by a Dickson plot). To clarify the basis of HNE-derived inhibition of poly(ADP-ribose) formation, we measured the influence of 1 and 10 μM HNE on NAD^+ hydrolysis (Table 3). Whereas the NAD^+ hydrolysis was inhibited by 20 or 25% in the case of 1 or 10 μM HNE respectively, the ADP-

Figure 2 Inhibition of isolated ADP-ribosyltransferase by HNE

The dependence of inhibition of poly(ADP-ribose) formation on HNE concentration is shown. Each data point represents the mean for four independent experiments with S.D. less than 7%. The inset is a Dickson plot, which was used to determine the K_i (about 4 μM).

Table 3 ADP-ribosyltransferase and NAD^+ -hydrolytic activity of isolated poly(ADP-ribosyl)transferase after HNE treatment

ADP incorporation was monitored by incorporation of [^{14}C]adenine, and NAD^+ hydrolysis was measured by HPLC. The values represent the mean for three experiments.

HNE concn. (μM)	ADP-ribosyltransferase activity (nmol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$)	(%)	NAD^+ hydrolysis (nmol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$)	(%)	Ratio*
0	107.3	100	138.3	100	1.3
1	81.1	75.6	111.6	80.7	1.4
10	29.9	27.8	104.0	75.2	3.5

* Ratio NAD^+ hydrolysis/protein-bound ADP-ribose [48].

ribosyltransferase activity was decreased by 25 or 75% respectively. Therefore the ratio of NAD^+ hydrolysis per mol of protein-bound ADP-ribose increases. This is a clear indicator of a drastic decrease of ADP-ribosyltransferase activity in comparison with NAD^+ hydrolysis (see [48]). Treatment of rabbit synovial fibroblasts with 1 or 10 μM HNE treatment leads to an inactivation in ADP-ribosyltransferase activity as shown by a decrease of the ADP-incorporation by 22 or 26% respectively.

DISCUSSION

Micromolar concentrations of HNE inhibit poly(ADP-ribose) synthesis in rabbit synovial fibroblasts treated with active oxygen. This decrease in the poly(ADP-ribose) level is not due to the impairment of XO by HNE, because neither rat liver nor milk XO showed a decrease in their activity during 30 min of incubation with HNE over a concentration range from 1 to 10 μM [38]. A possible function of HNE as a messenger of the primary free-radical event leading to the activation of the ADP-ribosyltransferase can be excluded, since HNE alone leads to no accumulation of poly(ADP-ribose) or stimulation of the ADP-ribosyltransferase. Inhibition of poly(ADP-ribose) synthesis is possibly due to a direct inhibition of the ADP-ribosyltransferase, as shown in the *in vitro* incubation of the isolated enzyme with HNE. The K_i estimated by a Dickson plot is about 4 μM , a

possible pathophysiological tissue concentration. Fatty acids are well-known inhibitors of the ADP-ribosyltransferase [39]; however, HNE exhibits a substantially stronger inhibitory effect on poly(ADP-ribose)ylation than any fatty acids tested. The direct inhibition of this nuclear enzyme may explain the effect of exogenous HNE in lowering poly(ADP-ribose) levels. This direct inhibition of the poly(ADP-ribose) transferase may also play a role during the treatment of rabbit synovial fibroblasts, as indicated by the decreased maximal activity of the enzyme after lysis of cells following HNE treatment. Poly(ADP-ribose) has a very short half-life of between 30 s and 3 min [40–42]. Following a 30 min treatment with active oxygen and HNE, a decrease in the poly(ADP-ribose) content occurs. This could be caused by an inhibition of poly(ADP-ribose) synthesis or by enhanced poly(ADP-ribose) degradation. Poly(ADP-ribose) is synthesized from NAD⁺ on histones and a few other nuclear proteins by poly(ADP-ribose) polymerase, a zinc-binding nuclear DNA-dependent enzyme [25]. We have demonstrated that HNE is able to decrease the NAD⁺ hydrolysis activity of ADP-ribosyltransferase at concentrations which inhibit the enzyme intracellularly. Richter and Meier reported previously an inhibition of intramitochondrial pyridine-nucleotide hydrolysis by HNE over the concentration range from 10 μ M to 50 μ M [43]. We found that the inhibition of NAD⁺ hydrolysis by HNE may also occur in the cell nucleus. This inhibition of ADP-ribosyltransferase is not only due to the decreased NAD⁺ hydrolysis, as demonstrated by a larger decrease in ADP-ribose transfer. This may be the result of additional modification of thiol groups of the ADP-ribosyltransferase by HNE, since it is known that the transferase is inhibited by SH-modifying agents [44].

Endogenous HNE generated by the X/XO oxidase system could be detected in the synovial cells in nanomolar concentrations. It is unlikely that HNE derived from the active-oxygen-generating X/XO system reaches concentrations in the synovial cell culture that inhibit poly(ADP-ribose) synthesis. Thus the endogenous HNE formed by lipid peroxidation in the synovial fibroblast culture does not interfere with the exogenous HNE.

Procollagen-gene transcription is controlled by the level of poly(ADP-ribose), an increase of poly(ADP-ribose) down-regulating the collagen gene transcription. The inhibition of ADP-ribosyltransferase, e.g. with 3-aminobenzamide or by depletion of NAD⁺, strongly stimulates procollagen mRNA transcription and, therefore, collagen synthesis [29]. Poly(ADP-ribose) synthesis in the nucleus is essential for efficient DNA repair and is mediated via activation of ADP-ribosyltransferase by DNA strand-breaks [45–48]. We could demonstrate in our experiments that HNE over a concentration range which occurs *in vivo*, e.g. in the synovial fluid of a patient with rheumatoid arthritis [19], inhibits the formation of poly(ADP-ribose) after treatment with active oxygen. We propose that HNE, formed in inflammatory tissues via lipid peroxidation, could inhibit poly(ADP-ribose) synthesis *in vivo* and the repair of the DNA damage initiated by the release of active oxygen.

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