Human tear lipocalin acts as an oxidative-stress-induced scavenger of potentially harmful lipid peroxidation products in a cell culture system

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Human tear lipocalin [lipocalin 1 (lcn-1); von Ebner's gland protein] is a member of the lipocalin superfamily that is known to bind an unusual variety of lipophilic ligands. Because of its properties and its tissue-specific expression it has been suggested that lcn-1 might act as a physiological protection factor of epithelia. Overexpression of lcn-1 under certain disease conditions supported such a function. However, experimental investigations into its exact biological role and its mode of expression were impeded because lcn-1 was previously found to be produced only in serous glands. To overcome this problem we therefore sought a cell line that produced lcn-1 endogenously. Using reverse-transcriptase-mediated PCR analysis we found expression of lcn-1 in the human teratocarcinoma-derived NT2 precursor cells. Under normal conditions the production of lcn-1 is low. However, treatment of the cells with H₂O₂ or FeSO₄, which typically induce lipid peroxidation, significantly enhanced

the expression of lcn-1. Binding studies revealed that arachidonic acid and several lipid peroxidation products including 7 β -hydroxycholesterol, 8-isoprostane and 13-hydroxy-9,11-octadecadienoic acid specifically bind to lcn-1. To investigate the physiological consequence of this observation we purified holo-(lcn-1) from culture medium and extracted the bound ligands. The presence of F₂-isoprostanes in the extracts obtained from the fractions containing lcn-1 indicates that these typical lipid peroxidation products are indeed ligands of the protein *in vivo*. These results support the idea that lcn-1 acts as a physiological scavenger of potentially harmful lipophilic molecules; lcn-1 might therefore be a novel member of the cellular defence against the deleterious effects of oxidative stress.

Key words: lipid binding, NT2 precursor cells, overexpression, reactive oxygen species.

INTRODUCTION

Human tear lipocalin [lipocalin 1 (lcn-1)], which is identical with von Ebner's gland protein, is a remarkable member of the lipocalin superfamily because it is known to bind a large variety of different classes of lipophilic ligands, including fatty acids, phospholipids, glycolipids, cholesterol, retinol and retinoic acid. In contrast with other lipocalins, many of these lipids are physiological ligands of lcn-1 [1,2]. Although originally isolated as a protein highly produced by the lacrimal and lingual salivary glands [3–5], it was afterwards found to be expressed by several other secretory tissues such as prostate [6], mucosal glands of the tracheobronchial tree [7], nasal mucosa [8] and sweat glands [3,9]. The precise biological function of lcn-1 has not been fully characterized and is still a matter of controversy. In human tear fluid it seems to be most important for the integrity of the tear film by removing lipids from the mucous surface of the eye to the liquid phase [10,11]. However, it has also been suggested to have a role in taste transduction or olfaction [5]. Interestingly, in addition to its lipid binding properties it displays additional activities *in vitro* that are very unusual among lipocalins, namely inhibition of cysteine proteinases and non-specific endonuclease activity [12,13]. Nevertheless, there is emerging evidence that its main function is to act as a general scavenger of lipophilic, potentially harmful molecules. It might therefore be protective towards tissues and cells [2].

A prerequisite for a protective function should be an induced production of lcn-1 under harmful conditions such as intoxication, infection, inflammation or other forms of cellular stress. Indeed, increased levels of lcn-1 were detected in bronchial secretions from patients with cystic fibrosis, a finding that was

correlated with increased levels of airway lipids [7]. In addition, it was reported recently that bronchoalveolar fluid from smokers contained higher overall levels of lcn-1 than that from non-smokers [14]. Consistent with these observations was our observation that the promoter region of *LCN1* contains several regulatory elements that are typically present in genes encoding acute-phase proteins [15]. However, systematic experimental investigations of the biological function, expression and inducible production of lcn-1 under the conditions mentioned above were hampered by the fact that lcn-1 has previously been found to be produced only by serous glands. We therefore sought a cultivable cell line with endogenous production of lcn-1 to address these questions in an experimental system.

Here we show that NT2 precursor cells, derived from a human teratocarcinoma, express low levels of lcn-1 mRNA. The production of lcn-1 was significantly enhanced by treatment with H₂O₂ or FeSO₄. Because both substances are well-known inducers of lipid peroxidation, we investigated a possible function of lcn-1 in the scavenging of lipid peroxidation products. Results from binding studies *in vitro* demonstrated that lcn-1 is able to bind several lipid peroxidation products. To verify that this interaction is of physiological relevance, we finally isolated holo-(lcn-1) from the medium of cells treated by H₂O₂ or FeSO₄, and analysed the bound ligands.

EXPERIMENTAL

Materials

8-Isoprostane and 13-hydroxy-9,11-octadecadienoic acid (HODE) were purchased from Cayman Chemicals (Ann Arbor,

Abbreviations used: AA, arachidonic acid; DAUDA, 11-(dansylamino)undecanoic acid; EIA, enzyme immunoassay; FCS, fetal calf serum; F₂-iP, F₂-isoprostanes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HODE, 13-hydroxy-9,11-octadecadienoic acid; lcn-1, lipocalin 1 (human tear lipocalin, von Ebner's gland protein); *LCN1*, gene encoding lcn-1; PG, prostaglandin; ROS, reactive oxygen species; RT-PCR, reverse-transcriptase-mediated PCR.

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MI, U.S.A.). 7 β -Hydroxycholesterol and arachidonic acid (AA) were obtained from Sigma (Deisenhofen, Germany). 4-Hydroxynonenal was purchased from Alexis Corp. (San Diego, CA, U.S.A.). The fluorescent fatty acid 11-(dansylamino)undecanoic acid (DAUDA) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Ethanol used for dissolving lipophilic ligands was of chromatographic grade (Merck, Darmstadt, Germany) and H₂O₂ was of analytical grade (Fluka Chemie AG, Buchs, Switzerland).

Cell culture

Human NT2/D1 precursor cells were obtained from Stratagene (La Jolla, CA, U.S.A.). Cells were propagated at 37 °C in DMEM/F-12 (Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 i.u./ml penicillin and 0.1 mg/ml streptomycin in 75 cm² cultural dishes under air/CO₂ (19:1). All experiments on the induction by H₂O₂ were performed in serum-free medium. At 1 day before induction, cells at 90% confluence were split and grown overnight to 70% confluence in medium containing 10% (v/v) FCS. Cells were washed twice in prewarmed PBS and shifted to serum-free medium for 16 h. H₂O₂ (0.1 mM) was added to the conditioned medium and cells were incubated for different periods as described for each experiment. For induction with iron, cells were grown overnight in medium containing 10% (v/v) FCS; 0.1 mM FeSO₄ was added and the cells were incubated for up to 1.5 h. The media were then collected and centrifuged to remove cell debris. The supernatants were either directly subjected to Western blot analysis and protein purification or stored at -80 °C.

RNA isolation, cDNA synthesis, semiquantitative reverse-transcriptase-mediated PCR (RT-PCR) and DNA sequencing

Total RNA from NT2 cells was extracted with TRISOLV[™] (Biotech), with a method based on that developed by Chomczynski and Sacchi [16]. Reverse transcription was performed by SuperScript[™] II reverse transcriptase (Life Technologies) with 3 μ g of total RNA and an oligo(dT) primer. After initial denaturation (3 min at 94 °C), cDNA was subjected to 35 cycles of PCR (denaturation at 94 °C for 40 s, annealing at 54 °C for 40 s and extension at 72 °C for 1 min) with primer lcn-1/1 (5'-TTGGAGGACTTTGAGAAAGC-3', derived from exon 5) and primer lcn-1/2 (5'-GCTGGATGGTGCCGTCC-3', derived from the 3' non-coding region of the lcn-1 cDNA) [3]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as an internal standard for semiquantitative PCR. Therefore a 295 bp GAPDH fragment was amplified from each first-strand sample by using primers GAPDH1 (5'-ACGTCGTGGAGTC-CACTG-3') and GAPDH2 (5'-GGGCCATCCACAGTCTTC-3'). After initial denaturation, 17 cycles of PCR (denaturation at 94 °C for 40 s, annealing at 54 °C for 40 s and extension at 72 °C for 1 min) were performed. Because the amplification of PCR products is linear only up to a limited number of cycles, we performed serial PCR for both lcn-1 (29–38 cycles) and GAPDH (14–23 cycles) to determine the linear range of amplification. For lcn-1 and GAPDH the linear ranges proved to be 35 and 17 cycles respectively. The quantity of PCR products was assessed with a DyNA Quant 200 Fluorimeter by using Hoechst 33258 dye.

For sequencing of the coding region of NT2-derived lcn-1 cDNA, a 620 nt fragment was amplified by PCR with primers lcn-1/3 (5'-AGCAAGCGACCTGTCAGG) and lcn-1/2 (5'-

GCTGGATGGTGCCGTCC-3'), corresponding to nt positions 19–36 and 621–638 respectively of the original lcn-1 cDNA sequence [3].

Western blot analysis of culture medium supernatant

A 25 μ l sample of supernatant was subjected to electrophoresis on precast 14%-(w/v)-polyacrylamide Tris/glycine gels (Novex, Groningen, The Netherlands); the proteins were electroblotted to nitrocellulose membranes. Blots were incubated with an antiserum raised against recombinant lcn-1 in rabbit. Alkaline-phosphatase-conjugated secondary antibodies (Sigma, St Louis, MO, U.S.A.) were used for detection.

Ligand binding studies

Lcn-1 used for binding studies was isolated from human tear fluid as described [3] and delipidated by chromatography on Lipidex 1000 [17]. Binding studies with AA were performed by internal tryptophan fluorescence quenching analysis, essentially as described for other lipocalins [18]. Experiments were performed in a Hitachi F4500 fluorescence spectrophotometer with slits set at 5 nm bandwidth at a constant temperature of 22 °C. Tryptophan fluorescence was excited at 295 nm and emission was recorded between 330 and 350 nm. For each measurement 250 μ l of protein solution (1–10 μ M) in 20 mM potassium phosphate/50 mM NaCl (pH 7.2) was used and mixed with 1 μ l of appropriately diluted ligand. A 10 mM stock solution of AA in ethanol was prepared gravimetrically and then diluted as required. The mixtures were incubated for 15 min at room temperature and the tryptophan fluorescence was measured in three independent experiments. The binding of lipid peroxidation products to lcn-1 was analysed by displacement of the fluorescent fatty acid DAUDA and performed in the instrument described above, except that the bandwidths for excitation and emission were 1 and 2 nm respectively. The excitation wavelength was 345 nm and fluorescence was measured at 490 nm. Samples of lcn-1 (2.5 μ M) were incubated with DAUDA (1.6 μ M) in the buffer as mentioned above; competitors (lipid peroxidation products) were added in increasing amounts. All lipids were dissolved in ethanol. In general, at the end of titration the ethanol concentration did not exceed 2%.

Purification of holo-(lcn-1) from culture medium supernatant

Cell culture medium (typically 100 ml) was filtered through Centriprep 50. The flow-through containing lcn-1 was adjusted to pH 6.5 and 10 mM NaCl, then subjected to anion-exchange chromatography on DEAE-Sephacel Fast Flow. Bound proteins were eluted with a linear NaCl gradient (0–500 mM) and the fractions were analysed by SDS/PAGE and Western blotting.

Determination of free and lcn-1-bound F₂-isoprostanes (F₂-iP) by enzyme immunoassay (EIA)

A commercially available F₂-iP EIA kit (Cayman Chemicals) was used for the detection of bound isoprostanes. In brief, lcn-1-containing fractions from anion-exchange chromatography were extracted three times with chloroform after alkaline hydrolysis; the organic phase was evaporated to dryness. The samples were dissolved in 1 ml of EIA buffer and the analysis was performed essentially as described by the supplier (Cayman Chemicals). The same assay was used for the determination of free F₂-iP in medium. The specificity of the antiserum was given as follows: 8-isoprostane 100%, 8-iso-prostaglandin (PG)_{F₂α}

20.55 %, 8-iso-PGE₂ 1.84 %, 2,3-dinor-8-iso-PGF_{2x} 1.7 %; 8-iso-PGE₁ 1.56 %, PGF_{1x} 0.71 %, PGF_{3x} 0.66 %, PGE₁ 0.39 %, PGD₂ 0.16 %, PGF_{2x} 0.14 %, 6-keto-PGF_{1x} 0.14 %, 2,3-dinor-6-keto-PGF_{1x} 0.09 %, thromboxane B₂ 0.08 %, 11-dehydrothromboxane B₂ 0.07 %, 11 β -PGF_{2x} 0.03 %, PGE₂ 0.02 %, 8-iso-15(R)-PGF_{2x} 0.02 %.

RESULTS

Expression of lcn-1 in NT2 cells

When several human cell lines, including fibroblasts, LNCaP cells, mammary gland cells and teratocarcinoma-derived NT2 cells, were screened for expression of lcn-1 by PCR with gene-specific primers, a specific PCR product was obtained from NT2 precursor cells (Figure 1A, lane 2), whereas all other cell lines tested were negative (results not shown). The human genome contains at least two different genes, *LCN1* and *LCN1b*, that potentially encode lcn-1 mRNA [9]. To find out whether the mRNA produced in NT2 cells is encoded by *LCN1* or by *LCN1b*, we performed a sequence analysis of the PCR products. The sequence of the amplified cDNA corresponded to the published sequence of tear lipocalin (GenBank® accession number M90424), which is encoded by *LCN1* [15]. Because lcn-1 is a typical secreted protein, we performed an immunoblot analysis of the cell culture supernatant. A faint band in the region of 18 kDa was stained by the lcn-1-specific antiserum (Figure 2, lane 2). These experiments clearly demonstrate a basal production of lcn-1 by NT2 cells, although at a low level under the conditions that we used.

Induction of lcn-1 expression by oxidative stress

Previous studies indicated that bronchoalveolar fluid from smokers contained higher levels of lcn-1 than that from non-smokers [14]. It is known that smoking induces the oxidative modification of lipids and an increase in circulation of lipid peroxidation products [19]. We therefore investigated whether

Figure 1 Agarose-gel electrophoresis of PCR products

Upper panels: RT-PCR analysis was performed with lcn-1-specific primers. (A) Results obtained from H₂O₂ induction. Lane 1, molecular mass marker; lane 2, RT-PCR product obtained from RNA of NT2 cells grown overnight in medium supplemented with FCS; lane 3, RT-PCR product from cells grown overnight in serum-free medium; lane 4, RT-PCR product from RNA of NT2 cells isolated 45 min after treatment with 0.1 mM H₂O₂; lane 5, negative control without cDNA. (B) Results obtained from FeSO₄ induction. Lane 1, molecular mass marker; lane 2, RT-PCR product obtained from RNA of NT2 cells grown overnight in medium supplemented with FCS; lane 3, RT-PCR product from RNA of NT2 cell isolated 45 min after treatment with 100 μ M FeSO₄; lane 4, negative control without cDNA. Lower panels: RT-PCR analysis of the housekeeping gene GAPDH was used as a positive control and as internal standard for quantification.

Figure 2 Western blot analysis of lcn-1 production by human NT2 cells

(A) Results obtained from H₂O₂ induction. Lane 1, molecular mass marker (masses indicated at the left); lane 2, 25 μ l of culture medium supernatant of NT2 cells grown overnight supplemented with FCS; lane 3, 25 μ l of culture medium supernatant of NT2 cells grown overnight in serum-free medium; lane 4, 25 μ l of culture medium supernatant, 45 min after the addition of 0.1 mM H₂O₂. (B) Results obtained from FeSO₄ induction. Lane 1, molecular mass marker; lane 2, 25 μ l of culture medium supernatant of NT2 cells grown overnight supplemented with FCS; lane 3, 25 μ l of culture medium supernatant, 45 min after the addition of 0.1 mM FeSO₄.

the low-level expression of lcn-1 was enhanced under conditions leading to lipid peroxidation. For this purpose we treated NT2 cells with H₂O₂, a classical inducer of lipid peroxidation. To avoid any influence of external lipid peroxidation products or antioxidants present in the serum, all experiments were performed in FCS-free medium. From Figure 2(A) (lane 4) it is evident that the addition of 0.1 mM H₂O₂ significantly increased the amount of lcn-1 present in the supernatant of the cell medium. A similar result was obtained at the mRNA level. Semiquantitative RT-PCR analysis indicated a 9-fold induction of lcn-1 mRNA after treatment with H₂O₂ (Figure 1A, lane 4) in comparison with the untreated cells. As determined by Trypan Blue exclusion assays, a slight decrease in viability of the treated cells was detected within 2 h of the addition of H₂O₂. However, between 80 % and 90 % of the cells remained attached to the culture dishes.

It should be mentioned that cells grown in medium supplemented with FCS showed a higher basal expression of lcn-1: 3-fold that of the cells grown in fully synthetic medium (Figure 1A, lanes 2,3). The same effect was also seen on Western blots (Figure 2A, lanes 2,3). It can be speculated that this result was due to some lipid peroxidation products present in the FCS. To investigate further the correlation of lcn-1 production and lipid peroxidation, we determined the amount of free F₂-iP, which are typical products of AA peroxidation and are used as markers *in vivo* for lipid peroxidation [20,21], in the culture medium. As determined by a specific EIA the concentration of free F₂-iP increased from 7.5 $\pm$ 1.1 (mean $\pm$ S.D.) pg/ml within the first 45 min to 14.8 $\pm$ 3.6 pg/ml at up to 2 h after the addition of H₂O₂.

To test whether other oxidants than H₂O₂ increased the expression of lcn-1, we treated the cells with 0.1 mM FeSO₄. Ferrous salts such as FeSO₄ are known to produce reactive oxygen species (ROS) at pH 7 via Fenton chemistry, thereby inducing harmful oxidative stress in cells [22]. Our results (Figures 1B and 2B) indicate that lcn-1 expression was also increased by Fe²⁺ ions, supporting the results obtained by H₂O₂ induction. From semiquantitative PCR an approx. 4-fold increase in lcn-1-specific mRNA was calculated.

Figure 3 Determination of the binding affinity of lcn-1 for AA by fluorescence titration experiments

Ligand concentration was plotted against the relative decrease in fluorescence. The set of data points was analysed by standard mathematical methods and a curve was fitted with non-linear hyperbolic regression.

Figure 4 Binding of the fluorescent fatty acid DAUDA to lcn-1 and competition by AA

The spectrum of DAUDA (5.2 μM) bound to lcn-1 (5.0 μM) shows enhancement and a blue shift (trace a), compared with DAUDA alone (trace c). The fluorescent enhancement is decreased by the addition of 4.8 μM AA (trace b).

Ligand-binding studies *in vitro*

To establish whether lcn-1 is capable of binding lipophilic compounds involved in or released by oxidative stress, we performed binding studies with fluorescence titration analysis or competitive displacement of the fluorescent fatty acid analogue DAUDA. Several members of the lipocalin superfamily, including lcn-1, show quenching of their intrinsic fluorescence owing to energy transfer from excited Trp when bound to their respective ligands [18,23,24]. Figure 3 shows the fluorescence data from binding experiments with AA with a 2 μM solution of delipidated protein mixed with the hydrophobic compound up to a final concentration of 10 μM . Our results clearly demonstrate that the AA-quenched fluorescence reached saturation, indicating

Figure 5 Competitive binding assays of lipid peroxidation products

Displacement of DAUDA (1.4 μM) from lcn-1 (5 μM) by increasing concentrations of an array of lipid peroxidation products: 7 β -hydroxycholesterol (■), IC_{50} 0.27 μM ; AA (●), IC_{50} 0.35 μM ; 8-isoprostane (▲), IC_{50} 0.94 μM ; HODE (▼), IC_{50} 1.1 μM ; hydroxynonenal (◆), IC_{50} 16.8 μM .

specific binding. An apparent dissociation constant (K_d) of 2.05 ± 0.22 μM (mean $\pm$ S.D., $n = 3$) was derived by mathematical analysis. To confirm these results we performed competitive binding experiments by displacement of the fluorescent fatty acid DAUDA. The characteristic emission spectrum of DAUDA permits the monitoring of displacement of a ligand as an indicator of relative binding affinity; thus the order of affinity of different ligands can be easily compared. It has been well established that the intensity of the fluorescence emission spectra of DAUDA shows enhancement and a blue shift when bound to proteins. The position of the emission maximum varies for different proteins but for lcn-1 it is known to be at 490 nm [24]. When DAUDA is displaced from lcn-1 there is a corresponding decrease in intensity and a red shift of the maximal emission peak. Figure 4 demonstrates that the fluorescence emission spectrum of DAUDA increases on binding to apo-lcn-1 and shows an emission maximum at 490 nm. This enhancement is decreased by the addition of AA, indicating the displacement of DAUDA by this ligand and confirming the specific affinity of lcn-1 for AA. The same assay was used to test whether typical lipid peroxidation products such as 8-isoprostane, HODE, 7 β -hydroxycholesterol or hydroxynonenal bind to lcn-1. The displacement curves for various concentrations of AA and several lipid peroxidation products, including 8-isoprostane, HODE and 7 β -hydroxycholesterol, are shown in Figure 5. All of these compounds were able to displace DAUDA. Our results therefore demonstrate clearly that they are potential ligands of lcn-1. Among the ligands tested, AA and 7 β -hydroxycholesterol showed the highest affinity; the lipid peroxidation products of AA, 8-isoprostane and HODE showed less affinity. This is in good agreement with previous studies demonstrating that the binding affinity of lcn-1 is inversely correlated with the hydrophobicity of the ligands [24]; the least soluble ligands therefore bind most strongly. We also tested binding of hydroxynonenal to lcn-1: as expected, it exhibited displacement of DAUDA but only at very high concentrations. Because DAUDA is known to bind within the calyx of lcn-1 [24], the results from the displacement experiments indicated that all ligands tested were bound entirely within the cavity of lcn-1.

Figure 6 Purification of holo-(lcn-1) and identification of F₂-iP as ligands *in vivo*

(A) Western blot analysis of fractions from ion-exchange chromatography. (B) Lipids from each fraction were extracted and analysed by a specific F₂-iP EIA. Each sample was assayed at two dilutions in duplicate; amounts of F₂-iP given are means of the duplicates.

Purification of holo-(lcn-1) and identification of bound F₂-iP

To establish whether the binding of lipid peroxidation products to lcn-1 was of relevance *in vivo*, we tried to isolate and purify a protein-lipid complex from the culture supernatant of H₂O₂-treated cells. It is known from gel-filtration studies that lcn-1 exists mainly in a dimeric or monomeric form. We therefore centrifuged the medium supernatant through Centrprep 50, to separate the bulk of high-molecular-mass proteins from lower-molecular-mass proteins. The flow-through containing lcn-1 was applied to an anion-exchange column and the fractions were analysed by SDS/PAGE and Western blotting. To isolate the bound lipids, each fraction was extracted with chloroform. It is known that lcn-1 is capable of binding a large number of different lipophilic ligands, which would probably result in a very complex ligand analysis. We therefore focused our interest on F₂-iP, which are typical lipid peroxidation products of AA. For the detection of lcn-1-bound F₂-iP a frequently used competitive immunoassay containing specific antibodies and acetylcholinesterase was applied [25,26]. As shown in Figure 6, an increase in the amount of F₂-iP extracted from the chromatography fractions was correlated with an increase in the amount of lcn-1 present in the same fractions. These results indicate that F₂-iP are ligands *in vivo* of the lcn-1 that is produced by the NT2 cells.

DISCUSSION

In the present study we have demonstrated that the human teratocarcinoma-derived NT2 precursor cells produce lcn-1. Previously, lcn-1 has been observed to be produced exclusively by acinar cells of the serous compartment within submucosal sero-mucinous glands of several organs [3–8]. Because these tissues are difficult to cultivate, their use in experimental studies is limited. Our result therefore has relevant implications for further investigations of the proposed multiple functions of lcn-1. Its importance to the human tear film became evident from recent studies [10,11,27]. However, its distribution in several

different tissues and its binding capacity for numerous lipophilic ligands [1,2,3–9] indicate a more general function. There is some evidence that lcn-1 production is induced by several factors such as inflammation, infection or toxic substances. Increased levels of lcn-1 were found in tracheal secretions of patients with cystic fibrosis, which was accompanied by bacterial infection and increased levels of airway lipids [7]. An increase in lcn-1 production was also found in bronchoalveolar fluid from smokers [14], supporting previous indications of enhanced production of lcn-1 in tear fluid from people exposed to industrial smoke [28]. These observations prompted us to investigate the role of oxidative stress in lcn-1 expression in the NT2 cell system. In the present study we found clear experimental evidence that lcn-1 production is induced by H₂O₂ and FeSO₄, both of which are classical inducers of oxidative stress by generating ROS. Numerous effects of ROS on biological molecules have been described, including damage to DNA, proteins and other macromolecules. Among them, lipid peroxidation is under the most intensive investigation because it gives rise to the production of harmful molecules such as lipid epoxides, lipid alkoxyl and peroxy radicals, unsaturated aldehydes and lipid hydroperoxides [29]. Lipid hydroperoxides, derived from unsaturated phospholipids, glycolipids and cholesterol by peroxidative reactions, can induce different cellular responses, ranging from the activation of stress signalling pathways and the induction of antioxidant enzymes to apoptotic or necrotic cell death [29,30].

The various defence mechanisms of cells against lipid peroxidation and other deleterious effects of oxidative stress can be principally divided into two types, both including inducible and constitutive systems. Primary defences are mainly preventive and rely on cytoprotection by the inactivation or scavenging of ROS and other redox reactions before lipid peroxidation takes place. This system includes enzymes such as superoxide dismutases, catalases or glutathione peroxidases, and also a variety of low-molecular-mass antioxidant compounds. A second line of defence involves the removal of lipid peroxidation products and subsequent repair reactions. However, this second line has not yet been well characterized. Several enzymes have been found to be involved in the intracellular detoxification of lipid peroxidation products [31–33]. In mechanistic terms, two pathways of cytoprotection have been proposed. The excision/reduction/repair pathway involves the consecutive action of phospholipase A₂, glutathione peroxidase and an acyltransferase, whereas the reduction/excision/repair pathway involves the action of phospholipid hydroperoxide glutathione peroxidase, phospholipase A₂ and acyltransferase. In addition, some other enzymes such as selenoperoxidase, which seems to be important for the overall disposal of lipid hydroperoxides, and lecithin:cholesterol acyltransferase have been proposed to be involved in the detoxification process. The results of our study suggest that lcn-1 might have a role in this protection system but is clearly acting on an extracellular level and is probably restricted to specific areas.

Our finding that AA and lipid peroxidation products bind to lcn-1 is new but not completely unexpected. Binding of AA was also observed with apolipoprotein D [34], another lipocalin member. However, binding of classical lipid peroxidation products such as 7 β -hydroxycholesterol, 8-isoprostane or HODE to a lipocalin has not previously been described.

Considering that lcn-1 is highly expressed in glands of mucosal tissues, which contain numerous channels, another aspect of the action of non-esterified fatty acids has to be discussed. Several studies have demonstrated that non-esterified fatty acids affect a wide variety of ion channels, either to inhibit their activity or to activate them [35–37]. Most interestingly, among channels in-

hibited by fatty acids there are gap channels in lacrimal gland cells and Cl^- channels in airways epithelia [38–40]. Because the same inhibitory fatty acids are potential ligands of lcn-1, a role for this protein in interfering with channel activity or in the protection of channels might be suggested.

The use of knock-out experiments in mice as the classical method of investigating the function of lcn-1 *in vivo* is not feasible owing to the lack of a murine lcn-1 counterpart [41,42]. Therefore the availability of a suitable experimental cell system will enable us to investigate further the proposed functions of lcn-1 by the use of targeted deletion or anti-sense knock-out of *LCN1* in the NT2 cells.

Finally, the results presented might contribute to an elucidation of the proposed function of lcn-1 as a factor involved in olfaction or taste transduction. Lcn-1 shows significant amino acid similarity to the rat odorant-binding protein OBP II [43] and the mouse pheromone-binding proteins VNSP I and II [44] and is highly expressed in nasal mucus and the lingual von Ebner's glands, which are intimately associated with taste buds of circumvallatae and foliatae papillae. It was therefore suggested that lcn-1 has a role in olfaction or taste transduction [5,45], although binding studies with several odorants and tastants failed to confirm lcn-1 as a carrier for any of these substances [41,46]. However, both our experimental evidence that lcn-1 expression is inducible by oxidative stress in NT2 cells and the fact that lcn-1 is produced by various tissues strongly argue against this specific function. Our results suggest lipid scavenging as the main physiological action of lcn-1. This is also in accord with its function, which has been shown to be relevant in human tear fluid. There are two reports demonstrating that in tear fluid lcn-1 increases the surface pressure of the aqueous solution while scavenging lipids from hydrophobic surfaces and delivering them to the aqueous phase of tears, thus maintaining the integrity of the tear film [10,47]. Furthermore, the homogeneous distribution of lcn-1 in the nasal fossae, even in areas far removed from the olfactory region [48], supports the idea of a scavenging function rather than that of an odour carrier or transducer.

Apart from lipid ligand binding, two other biochemical activities of lcn-1 have been found by studies *in vitro*, namely cysteine proteinase inhibition and non-specific endonuclease activity [12,13]. These findings might enhance the protective function of lcn-1 as a non-immunological defence against bacteria and viruses. However, the physiological relevance of these activities *in vitro* must be further investigated.

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