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Enhancement of low density lipoprotein catabolism by non-steroidal anti-inflammatory drugs in cultured HepG2 cells

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Abstract

Several clinical studies have shown that different types of non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the cholesterol content of atherosclerotic blood vessels. The mechanism of this reduction is not established. One possibility is that NSAIDs affect low density lipoprotein (LDL) catabolism. In this study, we investigated the effect of the NSAIDs, indomethacin, flufenamic acid, ibuprofen, acetaminophen, and also acetylsalicylic acid on LDL binding, cell-association and degradation in cultured hepatoma HepG2 cells. LDL was labelled with ^{125}I to study LDL catabolism. Furthermore, dextran sulphate, a substance that is known to release bound LDL from its receptors, was used to study LDL receptor activity. Reverse transcription-polymerase chain reaction was used to study the messenger RNA (mRNA) of LDL receptor. Our results show that flufenamic acid, indomethacin, and to a lesser extent ibuprofen, and acetaminophen increase LDL binding, cell-association, and degradation. Flufenamic acid was most potent and increased LDL catabolism by 50–70%, whereas acetylsalicylic acid had only a modest effect. Also, flufenamic acid and indomethacin were both found to increase the synthesis of mRNA of the LDL receptor with a subsequent increase of LDL receptor protein. We also investigated the effect of indomethacin on LDL binding in the presence of the 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase inhibitor, fluvastatin. We found that both indomethacin and fluvastatin had an additive up-regulatory effect on LDL receptor activity. In addition the effect of flufenamic acid on cell-associated LDL was examined in the presence of cyclosporine, which is known to decrease LDL catabolism. The results show that flufenamic acid can restore the inhibitory effect of cyclosporine. The study thus shows that NSAIDs enhance LDL catabolism due to increased synthesis of the mRNA for LDL receptor protein. This action might contribute to the lipid-lowering effect of NSAIDs.

Keywords: Cyclosporine; Flufenamic acid; Fluvastatin; Indomethacin; Low density lipoprotein; Peroxisome proliferator-activated receptor-γ

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used clinically as anti-inflammatory, anti-pyretic, and anti-rheumatic agents. They exert their effect by inhibiting cyclooxygenase activity with subsequent inhibition of the formation of prostaglandins, prostacyclins, and thromboxanes (Hochberg, 1989). They also induce a multitude of biological effects, like inhibition of polymorphonuclear leucocyte aggregation and activation (Hochberg, 1989), decreased production of monocyte derived inflammatory cytokines (Jiang et al., 1998), inhibition of the release of lysosomal enzymes and superoxide radicals from polymorphonuclear leucocytes and monocytes/macrophages (Hochberg, 1989). These effects play an important role in the development of atherosclerosis (Elneihoum et al., 1997). Several studies have shown that indomethacin or ibuprofen reduce the progression of atherosclerosis and lower plasma cholesterol levels in humans or laboratory animals (Foxworthy et al., 1993; Gansevoort et al., 1994). Other studies have shown that indomethacin lowers the cholesterol content in liver and atherosclerotic blood vessels (Dhawan et al., 1992; Stoller et al., 1993). The mechanism of these actions is, however, not known. In a previous study, we have shown that troglitazone, a new antidiabetic thiazolidinedione compound, increases low density lipoprotein receptor activity in cultured HepG2 cells (Al Rayyes and Florén, 1998). Troglitazone has been shown to bind and activate peroxisome proliferator-activated receptor gamma (PPARγ) (Park et al., 1997). A recent study has also shown that NSAIDs activate peroxi-

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some proliferator-activated receptors (PPARs) (Lehmann et al., 1997). It may therefore be hypothesised that NSAIDs enhance low density lipoprotein (LDL) catabolism. Accordingly, the aim of this study was to examine the effect of the NSAIDs indomethacin, flufenamic acid, ibuprofen, acetaminophen, and acetylsalicylic acid on LDL binding, cell-association, and LDL degradation. The hepatoma cell line, HepG2 was used, because this cell line is highly differentiated and shows many biochemical similarities with human hepatocytes in that it, for example, expresses functional LDL receptor.

2. Materials and methods

2.1. Drugs

Indomethacin, ibuprofen, acetylsalicylic acid, acetamidophenol, and flufenamic acid were bought from Sigma. A stock solution was prepared by dissolving the different compounds in ethanol, except acetylsalicylic acid, which was dissolved in dimethyl sulphoxide. Fluvastatin (dissolved in water) and cyclosporine (dissolved in ethanol) were obtained as powder from Sandoz Pharm, Basel, Switzerland. The same concentration of ethanol and dimethyl sulphoxide, which were used as solvents in the test groups, was added to the control groups.

2.2. Cell culture

The established hepatoblastoma cell line HepG2 was obtained from the American Tissue Type Culture Collection. The cells were grown at 37°C in 80-cm^2 flasks (Nunclon, Roskilde, Denmark) in 25 ml RPMI 1640 medium containing 10% fetal calf serum (Biological Industries, Israel) in humidified air with 5% CO_2.

The cells were trypsinized with 0.05% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) (Life Technologies) and placed in 9-cm^2 plastic Petri dishes (Nunclon) and grown for two days in 2 ml medium supplemented with 10% fetal calf serum. The medium was then changed to a new one containing 10% fetal calf serum for the cell-association and degradation experiments and 5% lipoprotein-deficient fetal calf serum for the binding and immunoblotting experiments. The cells were left to grow for one more day prior to the beginning of the experiment (at that time the cells became subconfluent, covering about 75% of the bottom of the dish). Lipoprotein-deficient fetal calf serum was prepared from fetal calf serum by ultracentrifugation, after changing the density of the fetal calf serum with potassium bromide to 1.215 kg/l, for 48 h at 320,000 g_{max} at 4°C. The density of the bottom fraction was changed to 1.25 kg/l and re-ultracentrifugated under the same conditions as above. The bottom fraction was then collected and used as lipoprotein-deficient fetal calf serum.

2.3. Lipoprotein isolation and labelling

Blood was drawn from normocholesterolemic subjects into Vacutainer tubes containing 0.084 ml 0.34 M EDTA (100 × 16 mm, Becton Dickinson, Rutherford, NJ). Thimerosal (Sodium ethylmercurithiosalicylate, Sigma) was added to a final concentration of 25 μM to inhibit proteolytic activity and bacterial growth.

Plasma was collected after centrifugation in a Coolspin 2 centrifuge and LDL was isolated by sequential preparative ultracentrifugation as described by Havel et al. (1955), using a Beckman, Optima XL-80K Ultracentrifuge.

A narrow density range (1.034 kg/l–1.054 kg/l) was used to prepare LDL for the experiments to minimize contamination of LDL with apolipoprotein E.

LDL was labelled with ^{125}I by the iodine monochloride method (McFarlane, 1958). Unbound ^{125}I was removed by chromatography on Sephadex G-25 columns PD-10 (Pharmacia, Uppsala, Sweden) followed by extensive dialysis against 0.9% NaCl, 1 mM EDTA and 0.03 M KI, and further dialysis against 0.9% NaCl containing 1 mM EDTA. The specific activity of ^{125}I-LDL was 545–845 cpm/ng LDL protein.

2.4. Cell-associated and degraded ^{125}I-LDL assays

HepG2 cells were cultured to subconfluency, washed with 1 ml phosphate-buffered saline (PBS) 3 times, incubated in 2 ml RPMI 1640 (Gibco, Life Technologies) containing 0.5% human serum albumin, (Fraction V, Sigma) and various concentrations of indomethacin, flufenamic acid, ibuprofen, acetaminophen, acetylsalicylic acid, and cyclosporine (1–40 μg/ml) according to the protocol of the experiments. After incubation for a limited time at 37°C, the medium was aspirated, and the cells were washed with PBS and new RPMI medium was added together with ^{125}I-LDL (0.6 μg LDL protein/ml) and the cells were incubated for a limited time according to the protocol of the experiments. The medium was then taken for subsequent determination of LDL degradation, which was measured as non-iodine trichloroacetic acid-soluble radioactivity (Bierman et al., 1974). The cells were then washed with 1 ml PBS 3 times and scraped off with 2 ml 0.5 M NaOH to be measured in a 1470 WIZARD automatic gamma counter (Wallac, Turku, Finland) for determination of cell-associated LDL.

2.5. LDL binding assay

Because internalization of ligands by receptor-mediated endocytosis is inhibited at 4°C, the binding of LDL to HepG2 cells was examined at this temperature. Subconfluent cells were washed with 1 ml PBS 3 times and then incubated for 24 h in 2 ml RPMI 1640 containing 0.5% human serum albumin and different concentrations of indomethacin, flufenamic acid, ibuprofen, acetaminophen,

and acetylsalicylic acid (2.5–40 μg/ml) according to the protocol of the experiments. The cells were also incubated with 10 ng/ml fluvastatin in the experiment with this compound. After that, the cells were washed 3 times with 1 ml ice-cold PBS followed by pre-cooling for 20 min at 4°C in 2 ml ice-cold RPMI medium. After addition of ^{125}I-LDL (600 μg/ml) the cells were incubated for 2 h at 4°C, then washed 3 times with 1 ml ice-cold PBS. 2 ml cold PBS containing 10 mg/ml dextran sulphate (Pharmacia Biotech., Uppsala, Sweden) was added and the cells were subjected to a second incubation for 1 h at 4°C in a rotatory shaker at 60 rpm. Medium was then aspirated and subjected to measurement of radioactivity (Salter et al., 1986).

2.6. Cell membrane protein preparation

The cells were trypsinized and placed in 9-cm^2 plastic Petri dishes (Nunclon) and grown for two days in 2 ml medium supplemented with 10% fetal calf serum. The medium was then changed to a new one containing 5% lipoprotein-deficient fetal calf serum, and the cells were left to grow for 24 h more (the subconfluent state). Subconfluent cells were washed with 1 ml PBS 3 times and then incubated for 24 h in 2 ml RPMI 1640 containing 0.5% human serum albumin and 40 μg/ml flufenamic acid and indomethacin. The cells were then washed with cold PBS and solubilized in 100 μl 1.6% Triton X-100, 5 M urea, 0.3 mM leupeptin, and 1.5 mM phenylmethylsulphonyl fluoride, and incubated on ice for 30 min, then scraped off and homogenized in tissue homogenizer. The resulting suspension was centrifuged in a Beckman Airfuge at 100,000 *g* for 15 min and the supernatant from 6 dishes for every group was pooled and stored in liquid nitrogen for subsequent immunoblotting (Srivastava et al., 1995).

2.7. LDL receptor protein quantification

LDL receptor protein quantification was done as described in a previous study with some modifications (Srivastava et al., 1995). The blocking buffer consisted of 10 mM Tris, 0.15 M NaCl, 0.5% Tween 20 and 3% gel fish (Sigma). The concentration of the primary mouse monoclonal anti-human LDL receptor antibody (clone C7) RPN 537 was 5 μg/ml (Amersham Life Science, Buckinghamshire, England). After incubation of the membrane with the primary antibody, it was incubated for 4 h with alkaline-phosphatase-conjugated rabbit anti-mouse immunoglobulins (Code No. D 0314, Dako, Denmark) and then washed. Colour development was performed by using alkaline phosphatase conjugate substrate kit (catalogue no. 170-6432, Bio Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547). The density of the bands was then scanned in a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). The densities were analyzed by ImageQuant software, Molecular Dynamics.

2.8. RNA isolation

Total RNA was prepared from HepG2 cells by a single-step guanidinium thiocyanate extraction method (Ausubel et al., 1993) and stored at −80°C until LDL receptor messenger RNA (mRNA) was quantified by reverse transcription-polymerase chain reaction. The quantity of RNA was estimated by spectrophotometric readings at 260 and 280 nm.

2.9. Reverse transcription-polymerase chain reaction and quantitative analysis of mRNA

Reverse transcription-polymerase chain reaction and quantitative analysis of mRNA were performed exactly as previously described (Al Rayyes et al., 1997).

2.10. Protein determination

The protein content of LDL and cells was determined using the Lowry assay with human serum albumin as standard (Lowry et al., 1951).

2.11. Statistical analysis

The differences of the means of experimental results were analyzed for statistical significance with Independent-Samples Student's *t* test or with one-way analysis of variance (ANOVA) and Duncan's multiple range test with

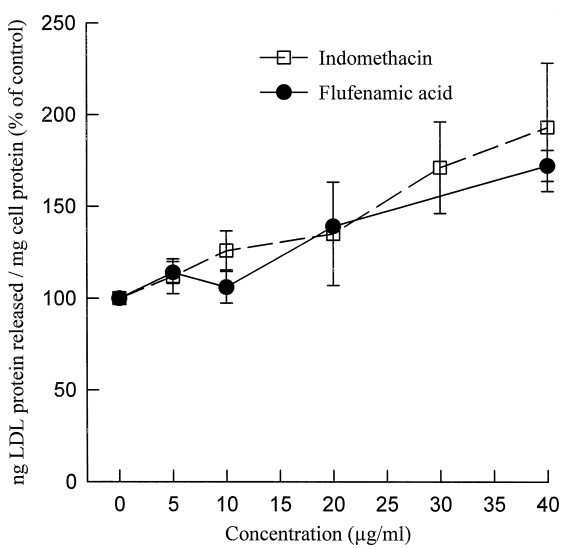

Fig. 1. Effect of various concentrations of indomethacin and flufenamic acid on ^{125}I-LDL binding in HepG2 cells. The cells were incubated with different concentrations of indomethacin and flufenamic acid (5–40 μg/ml) for 24 h, then washed with PBS, and prechilled for 20 min at 4°C. ^{125}I-LDL (600 μg/ml) was added and the cells were then incubated for 2 h at the same temperature. The results are statistically significant at all points with $P < 0.05$. Each point represents mean ± S.D. for three dishes obtained from one of four similar experiments after normalization the control data to 100%.

a significance level of 0.05 (Afifi and Azen, 1997). SPSS for Windows, release 6.0, was used for the statistical calculations (Norusis, 1993).

3. Results

3.1. Effect of indomethacin and flufenamic acid on LDL binding

Fig. 1 shows that indomethacin concentration dependently increased the binding of ^{125}I-LDL to HepG2 cells. At 10 μg/ml, indomethacin increased LDL binding by 33% ($P < 0.02$) and at 40 μg/ml it increased the binding by 93% ($P < 0.01$). The mean percentage increase in binding induced by indomethacin in four experiments, after normalization of the control data to 100%, was 127 ± 6% ($P < 0.001$) at 10 μg/ml and 161 ± 24% at 40 μg/ml ($P < 0.07$) (mean ± S.D., $n = 4$). Fig. 2 shows the time dependency of the effect of indomethacin on LDL binding. The maximum effect was observed after 12 h, when binding induced by indomethacin surpassed that of control cells by 63% ($P < 0.005$). The mean percentage increase in LDL binding of the three experiments after 12 h (after normalization of the control data to 100%) was 155 ± 9.6% ($P < 0.01$, mean ± S.D., $n = 3$), and after 24 h it was 127 ± 11% ($P < 0.05$). Fig. 1 also shows that flufenamic acid at 5 μg/ml increased LDL binding by 14 ± ($P < 0.06$), and at 40 μg/ml it increased LDL binding by 72% ($P < 0.006$).

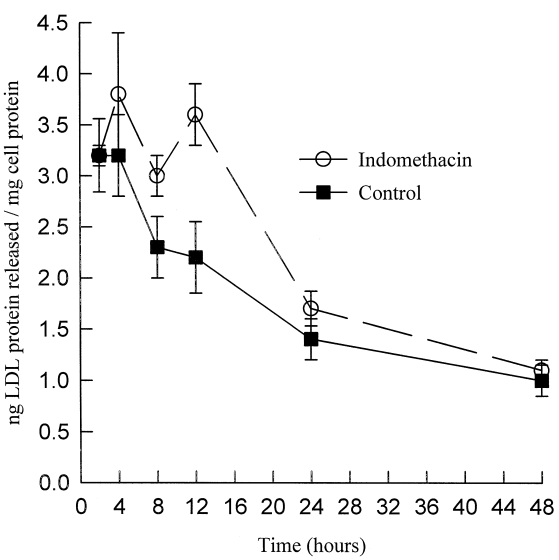

Fig. 2. Effect of indomethacin (30 μg/ml) on ^{125}I-LDL binding at different time periods (2–48 h). The cells were preincubated with indomethacin for 2–48 h, then washed with PBS, and prechilled for 20 min at 4°C. ^{125}I-LDL (600 μg/ml) was added and the cells were then incubated for 2 h at the same temperature. Each point represents mean ± S.D. for three dishes obtained from one of three similar experiments.

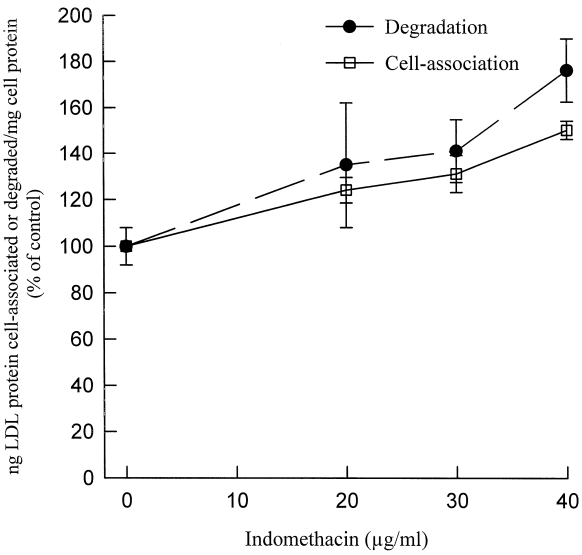

Fig. 3. Effect of indomethacin on cell-associated and degraded ^{125}I-LDL. The cells were incubated with different concentrations of indomethacin (20–40 μg/ml) for 24 h, washed with PBS, and incubated in 2 ml RPMI medium together with labelled LDL at 37°C for 4 h. The medium was then taken to measure degradation and the cells were then washed with PBS and scraped off in 0.5 M NaOH to measure cell-associated LDL. Each point represents mean ± S.D. for three dishes. The results are from one of four similar experiments.

3.2. Effect of indomethacin and flufenamic acid on cell-associated and degraded LDL

Fig. 3 shows that indomethacin at concentrations from 20–40 μg/ml significantly increased LDL cell-association and degradation. At 20 μg/ml the cell-association was increased by 24% ($P < 0.01$) and the degradation by 34% ($P < 0.01$). At 40 μg/ml the cell-association was in-

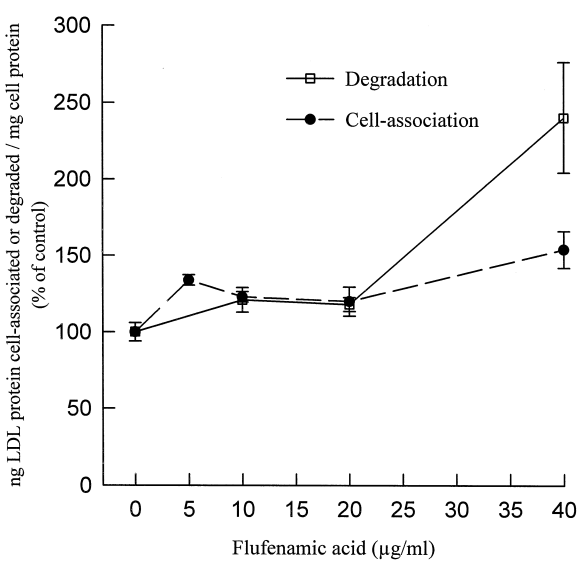

Fig. 4. Effect of different concentrations of flufenamic acid on LDL cell-association and degradation. The cells were preincubated with different concentrations of flufenamic acid (5–40 μg/ml) for 24 h, and then degradation and cell-association were measured as described in Section 2. The results are from one of three similar experiments.

creased by 50% ($P < 0.001$) and degradation by 77% ($P < 0.01$). Flufenamic acid at 10 μg/ml increased LDL degradation by 21% ($P < 0.02$); at 40 μg/ml degradation was increased by 140% ($P < 0.02$) and cell-association by 54% (Fig. 4).

3.3. Comparison between the different non-steroidal anti-inflammatory drugs on LDL binding, cell-association, and degradation

Fig. 5A shows the mean percentages of LDL binding in three different experiments with indomethacin, flufenamic acid, ibuprofen, acetaminophen, and acetylsalicylic acid. Flufenamic acid increased LDL binding by 50% ± 18 ($P < 0.04$, mean ± S.D., $n = 3$) and indomethacin increased LDL binding by 37% ± 10 ($P < 0.02$, mean ± S.D., $n = 3$). As shown in Fig. 5B, flufenamic acid increased LDL degradation by 50% ($P < 0.007$) and indomethacin by 43% ($P < 0.02$). This figure also shows that acetylsalicylic acid increased LDL degradation by 20% ($P < 0.03$). Cell-associated ^{125}I-LDL was increased by flufenamic acid to 72% ($P < 0.001$), and by indomethacin to 36% ($P < 0.006$).

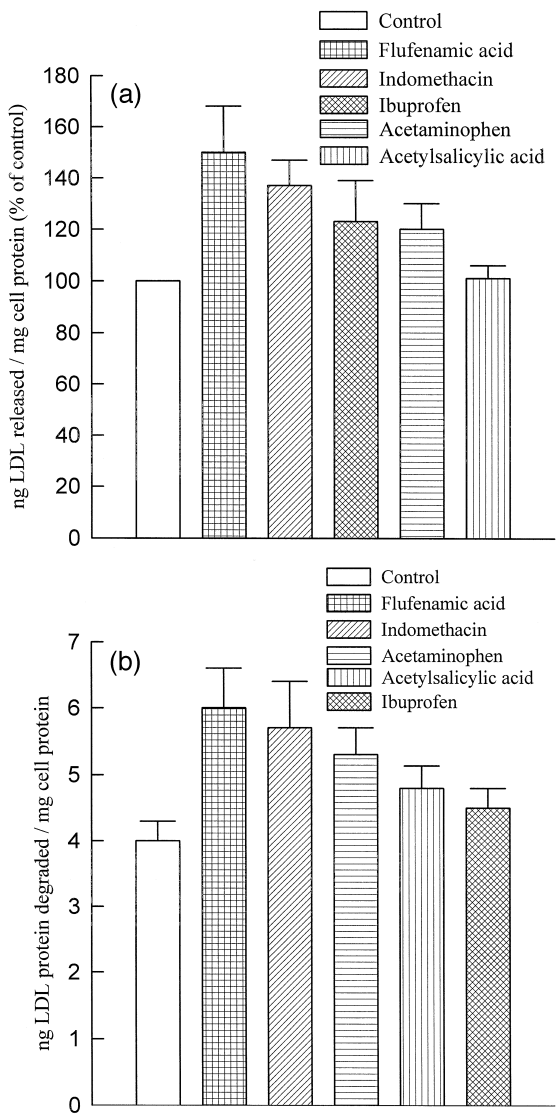

Fig. 5. (A) Effect of different NSAIDs on LDL binding. The cells were pre-incubated with 40 μg/ml of flufenamic acid, indomethacin, ibuprofen, acetaminophen, and acetylsalicylic acid for 24 h, and then the binding was measured as described in Section 2. The results are the mean percentage of three similar experiments after normalization of the control data to 100%. (B) Effect of different NSAIDs on the degradation of LDL. The cells were pre-incubated with 40 μg/ml of flufenamic acid, indomethacin, ibuprofen, acetaminophen, or acetylsalicylic acid for 24 h, and then degradation was measured as described above. The results are from one of three similar experiments.

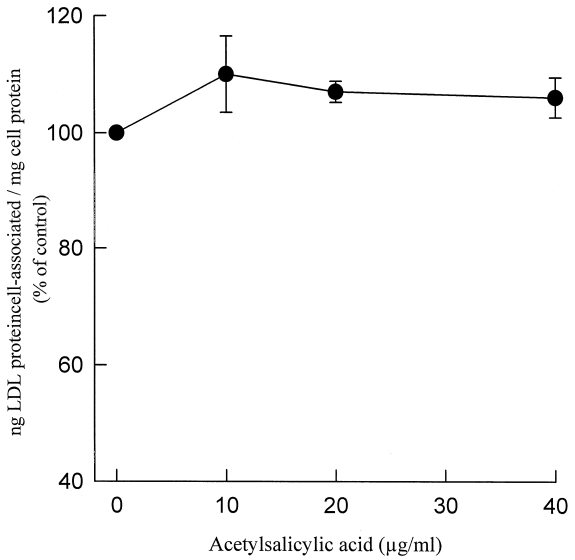

Fig. 6. Effect of acetylsalicylic acid on LDL binding. The cells were pre-incubated with various concentrations of acetylsalicylic acid (10–40 μg/ml) for 24 h, and then washed with PBS and prechilled for 20 min at 4°C. ^{125}I-LDL (600 μg/ml) was added and the cells were then incubated for 2 h at the same temperature. The results presented here are mean of three similar experiments after normalization of the control data to 100%.

3.4. Effect of acetylsalicylic acid on cell-associated ^{125}I-LDL

The effect of acetylsalicylic acid on cell-associated LDL was tested. Fig. 6 shows the mean percentage of three experiments after normalization of the control data to 100%. Acetylsalicylic acid at 10 μg/ml increased LDL cell-association by 10 ± 6.5% ($P < 0.05$, mean ± S.D., $n = 3$), at 20 μg/ml it increased the cell-association by 7 ± 1.8% ($P < 0.006$), and at 40 μg/ml by 6 ± 3.4 ($P < 0.03$).

3.5. Effect of indomethacin on LDL binding in the presence of the HMG CoA reductase inhibitor fluvastatin

Table 1 shows the combined effect of indomethacin and fluvastatin on LDL binding. Fluvastatin alone at 10 ng/ml increased LDL binding by 32%, while indomethacin at 30 μg/ml increased LDL binding by 18%, but both in com-

Table 1
The combined effect of indomethacin and fluvastatin on ^{125}I-LDL binding

	Fluvastatin(ng/ml)			
	0 ng/ml		10 ng/ml	
	ng LDL/mg cell protein	Relative %	ng LDL/mg cell protein	Relative %
Indomethacin (μg/ml)				
0	2.2 ± 0.4	100	2.9 ± 0.26	132
30	2.6 ± 0.24	118	3.6 ± 0.54	164
40	3.1 ± 0.4	141	4 ± 0.3	182

The cells were preincubated with fluvastatin (10 ng/ml) and indomethacin (30 and 40 μg/ml) either alone or in combination for 24 h, then washed with PBS and prechilled for 20 min at 4°C. ^{125}I-LDL (600 μg/ml) was added and the cells were then incubated for another 2 h at the same temperature. Data are expressed as means ± S.D. for 3 dishes. One-way ANOVA and Duncan's multiple range test ($\alpha = 0.05$) showed significant differences between the means of all test and control cells. The results are from one of three similar experiments.

bination increased LDL binding by 64%. Also indomethacin at 40 μg/ml increased LDL binding by 41%, while in the presence of 10 ng/ml fluvastatin it increased binding by 82%. One-way ANOVA and Duncan's multiple range test ($\alpha = 0.05$) showed significant differences between the means of all test and control cells.

3.6. Effect of flufenamic acid on cell-associated LDL in the presence of cyclosporine

To induce down-regulation of LDL receptor activity, the cells were pre-treated with 1 μg/ml cyclosporine for 24 h, then washed and incubated for another 24 h with either cyclosporine alone or with cyclosporine (1 μg/ml) and flufenamic acid (40 μg/ml). Fig. 7 shows that cyclosporine decreased cell-associated LDL by 20%. Flufenamic acid alone increased cell-associated LDL by 90%, and in the presence of cyclosporine it increased cell-associated LDL by 49%. One-way ANOVA and Duncan's multiple range test ($\alpha = 0.05$) showed significant differences between the means of all test and control cells.

3.7. Effect of flufenamic acid and indomethacin on the expression of LDL receptor mRNA, and synthesis of LDL receptor protein.

The yield of total RNA prepared from HepG2 cells was 63 ± 12 (mean ± S.D.) μg/mg total cellular protein. As shown in Fig. 8, flufenamic acid at a concentration of 40 μg/ml significantly increased the expression of mRNA for LDL receptor by 90% ($P < 0.001$). Indomethacin increased the expression of mRNA by 69% ($P < 0.02$). Flufenamic acid increased LDL receptor protein by 25% and indomethacin by 14%.

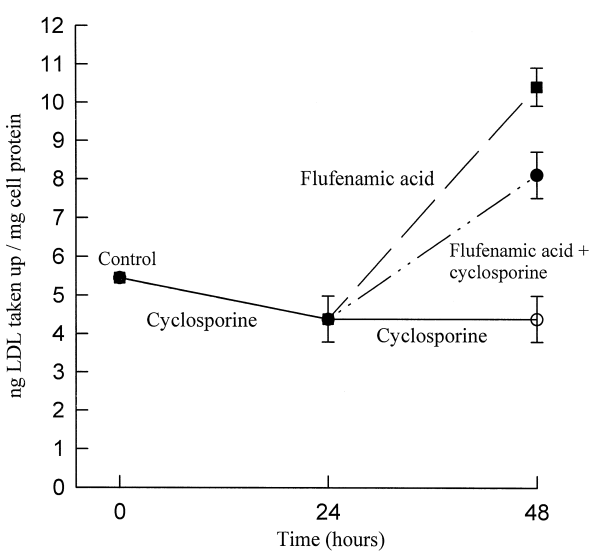

Fig. 7. Effect of flufenamic acid on cell-associated LDL in the presence of 1 μg/ml cyclosporine. The cells were pre-treated with cyclosporine for 24 h, and then washed with PBS. New RPMI medium containing human serum albumin was added together with cyclosporine (1 μg/ml) alone or cyclosporine and flufenamic acid (40 μg/ml). One-way ANOVA and Duncan's multiple range test ($\alpha = 0.05$) showed significant differences between the means of all test and control cells.

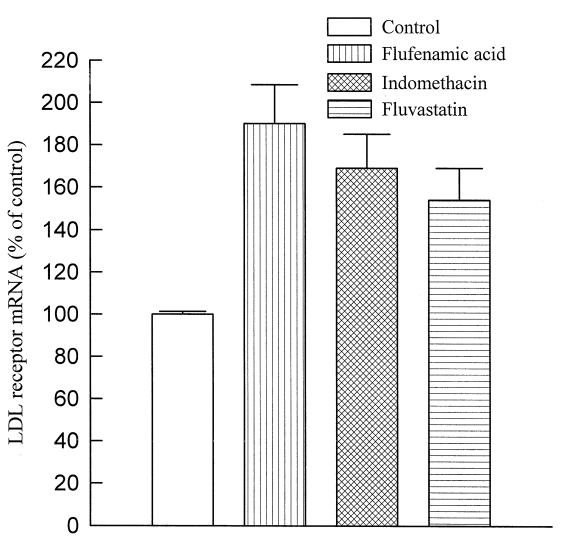

Fig. 8. Effect of flufenamic acid and indomethacin on the expression of LDL receptor mRNA. The values are expressed as percentages of the level of LDL receptor mRNA in non-treated cells. The results are from one of three similar experiments. Each point represents mean ± S.D. for five dishes.

4. Discussion

This study was undertaken to investigate the effect of NSAIDs which are commonly used as analgesic, anti-inflammatory, anti-pyretic, and anti-rheumatic agents, on LDL catabolism. Previously, we have shown that troglitazone increases LDL catabolism in cultured hepatoma cells, HepG2 cells (Al Rayyes and Florén, 1998). Troglitazone is a new anti-diabetic agent. It is one of the thiazolidinediones compounds which have been developed for insulin-resistant diabetes mellitus (Kraegen et al., 1989; Iwanishi and Kobayashi, 1993). These compounds bind and activate PPARγs (Park et al., 1997), which belong to the PPAR superfamily of nuclear hormone receptors, acting as ligand-activated transcription factors that modulate mainly the transcription of genes involved in fatty acid metabolism (Lefebvre et al., 1997; Vidal-Puig et al., 1997). NSAIDs have previously been shown to potently activate PPARs (Lehmann et al., 1997). Several studies have shown that NSAIDs also have a lowering effect on cholesterol levels in humans and experimental animals. Indomethacin can lower the cholesterol content of atherosclerotic blood vessels and liver in monkeys and rabbits (Dhawan et al., 1992; Stoller et al., 1993), and when combined with ACE inhibitors, indomethacin lowers blood cholesterol levels in humans (Gansevoort et al., 1994). Accordingly, our hypothesis was that NSAIDs could also have an up-regulatory effect on LDL catabolism. The effect of indomethacin, flufenamic acid, ibuprofen, acetaminophen, and acetylsalicylic acid on LDL catabolism was thus investigated. We found that various NSAIDs, particularly flufenamic acid and indomethacin, increased LDL binding, cell-association, and degradation as well as LDL receptor mRNA levels. Flufenamic acid increased LDL catabolism by approximately 70% and indomethacin increased it by approximately 35%, while acetylsalicylic acid had a modest effect. We interpret these findings to indicate an increase in LDL receptor gene expression, with subsequently increased mRNA formation and LDL receptor protein synthesis and activity. The mechanism of induction of LDL receptor activity by NSAIDs needs to be investigated; however, one can speculate that it could be due to an increase in the expression of PPARs, because NSAIDs can increase the expression of these nuclear receptors, which are also implicated in lipoprotein metabolism (Schoonjans et al., 1997).

Furthermore, our results together with the above-mentioned effects of NSAIDs point to the potential use of these drugs in the management of atherosclerosis. In this context, it is of interest that indomethacin and the 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase inhibitor, fluvastatin, exerted additive effects on LDL receptor activity. This additive effect could be of benefit for lowering circulating LDL-cholesterol levels in patients treated with both HMG CoA reductase inhibitors and NSAIDs. Previously we showed that the immunosuppressant drug, cyclosporine decreased LDL catabolism by down-regulating LDL receptor activity (Al Rayyes et al., 1996). Therefore we now investigated the effect of flufenamic acid on cell-associated LDL in the presence of cyclosporine. The results show that flufenamic acid can restore the inhibitory effect of cyclosporine on cell-associated LDL, but cyclosporine has a residual effect on the efficiency of flufenamic acid for restoring LDL receptor activity. The results concerning the effect of NSAIDs and troglitazone (Al Rayyes and Florén, 1998) on LDL receptor activity might be interpreted as suggesting that PPARs activate the peroxisome proliferator response element on genes involved in LDL catabolism. However, confirmation of this hypothesis requires an experimental design involving down-regulation of PPAR. Should the hypothesis be valid, it might provide a basis for defining new goals for the treatment of hypercholesterolemia.

In summary this study shows that the NSAIDs, especially flufenamic acid and indomethacin, increase LDL binding, cell-association, and degradation by increasing the expression of the mRNA of LDL receptor protein in HepG2 cells.

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References

Afifi, A.A., Azen, S.P., 1997. Statistical analysis, A computer oriented approach, 2nd edn., Academic Press, New York.

Al Rayyes, O., Florén, C.-H., 1998. Troglitazone up-regulates low density lipoprotein receptor activity in HepG2 cells. Diabetes 47, 1193–1198.

Al Rayyes, O., Wallmark, A., Florén, C.-H., 1996. Cyclosporine inhibits catabolism of low-density lipoproteins in HepG2 cells by 25%. Heptology 24, 613–619.

Al Rayyes, O., Wallmark, A., Florén, C.-H., 1997. Additive inhibitory effect of hydrocortisone and cyclosporine on low-density lipoprotein receptor activity in cultured HepG2 cells. Hepatology 26, 967–971.

Ausubel, M.A., Bernt, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1993. Preparation and analysis of RNA. Current protocols in molecular biology. Green Publishing Associates and Wiley, Cambridge, MA, 1:4.0.1–4.10.9.

Bierman, E.L., Stein, O., Stein, Y., 1974. Lipoprotein uptake and metabolism by aortic smooth muscle cells in tissue culture. Circ. Res. 35, 136–150.

Dhawan, V., Ganguly, N.K., Majumdar, S., Chakavarti, R.N., 1992. Effect of indomethacin on serum lipids, lipoproteins, prostaglandins and the extent and severity of atherosclerosis in rhesus monkeys. Can. J. Cardiology 8, 306–312.

Elneihoum, A.M., Falke, P., Hedblad, B., Lindgarde, F., Ohlsson, K.,

1997. Leukocyte activation in atherosclerosis: correlation with risk factors. Atherosclerosis 131, 79–84.

Foxworthy, P.S., Perry, D.N., Eacho, P.I., 1993. Induction of peroxisomal β-oxidation by nonsteroidal anti-inflammatory drugs. Toxicol. Appl. Pharmacol. 118, 271–274.

Gansevoort, R.T., Heeg, J.E., Dikkeschei, F.D., De Zeeuw, D., De Jong, P.E., Dullaart, R.P.F., 1994. Symptomatic antiproteinuric treatment decreases serum lipoprotein (a) concentration in patients with glomerular proteinuria. Nephrol. Dial. Transplant. 9, 244–250.

Havel, R.J., Eder, H.A., Bragdon, J.H., 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34, 1345–1353.

Hochberg, M.C., 1989. NSAIDs mechanisms and pathways of action. Hosp. Pract. 15, 185–198.

Iwanishi, M., Kobayashi, M., 1993. Effect of pioglitazone on insulin receptors of skeletal muscles from high-fat-fed rats. Metabolism 42, 1017–1021.

Jiang, C., Ting, A.T., Seed, B., 1998. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Kraegen, E.W., James, D.E., Jenkins, A.B., Chisholm, D.J., Storlien, L.H., 1989. A potent in vivo effect of ciglitazone on muscle insulin resistance induced by high fat feeding of rats. Metabolism 38, 1089–1093.

Lefebvre, A.M., Peinado-Onsurbe, J., Leitersdorf, I., Briggs, M.R., Paterniti, L.R., Fruchart, J.C., Fievet, C., Auwerx, J., Staels, B., 1997. Regulation of lipoprotein metabolism by thiazolidinediones occurs through a distinct but complementary mechanism relative to fibrates. Arterioscler. Thromb. Vasc. Biol. 17, 1756–1764.

Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406–3410.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurements with folin phenol reagents. J. Biol. Chem. 193, 265–275.

McFarlane, A.S., 1958. Efficient trace-labelling of proteins with iodine. Nature 182, 53.

Norusis, M.J., 1993. SPSS for Windows, rel. 6.0. SPSS, Chicago.

Park, K.S., Ciaraldi, T.P., Abrams-Carter, L., Mudaliar, S., Nikoulina, S.E., Henry, R.R., 1997. PPAR-γ gene expression is elevated in skeletal muscle of obese and type II diabetes subjects. Diabetes 46, 1230–1234.

Salter, A.M., Saxton, J., Brindley, D., 1986. Characterization of the binding of human low density lipoprotein to primary monolayer cultures of rat hepatocytes. Biochem. J. 240, 549–557.

Schoonjans, K., Martin, G., Staels, B., Auwerx, J., 1997. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr. Opin. Lipidol. 8, 66–159.

Srivastava, R.A.K., Ito, H., Hess, M., Srivastava, N., Schonfeld, G., 1995. Regulation of low density lipoprotein receptor gene expression in HepG2 and Caco2 cells by palmitate, oleate, and 25-hydroxycholesterol. J. Lipid. Res. 36, 1434–1446.

Stoller, D.K., Ground, C.B., Michalek, V., Buchwald, H., 1993. Reduction of atherosclerosis with nonsterolidal anti-inflammatory drugs. J. Surg. Res. 54, 7–11.

Vidal-Puig, A.J., Considine, R.V., Jimenez-Linan, M., Werman, A., Pories, W.J., Caro, J.F., Flier, J.S., 1997. Peroxisome proliferator-activated receptor gene expression in human tissues. Effect of obesity, weight loss, and regulation by insulin and glucocorticoids. J. Clin. Invest 99, 2416–2422.