# Altered signal pathway in granulocytes from patients with hypercholesterolemia

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**Abstract** **In the present study the signal transduction of the formyl-Met-Leu-Phe receptor was studied in granulocytes obtained from control subjects and patients with elevated low density lipoprotein levels. According to our results, 10 nM formyl-Met-Leu-Phe in control cells activates phospholipase C inducing a pronounced inositol phosphate production followed by a $Ca^{2+}$ signal from intracellular pools. The pertussis toxin-sensitive $O_2^-$ generation and leukotriene synthesis were moderate. In contrast, in granulocytes from hypercholesterolemic patients, formyl-Met-Leu-Phe triggered an intensive pertussis toxin-insensitive oxidative burst and leukotriene synthesis. The inositol trisphosphate and $Ca^{2+}$ signals were decreased significantly in granulocytes of hypercholesterolemic patients and seem to be dependent on the extracellular $Ca^{2+}$ content. Furthermore, in the resting granulocytes of hypercholesterolemic patients the $[Ca^{2+}]i$ and the membrane-bound protein kinase C activity were higher than in controls, the time of normalization after the low $Ca^{2+}$ signal was delayed, while the membrane fluidity was decreased. Our results suggest that in these ex vivo experiments, the high level of circulating low density lipoprotein in patients can affect the membrane composition of granulocytes leading to altered signal transduction by the formyl-Met-Leu-Phe receptor, to altered $Ca^{2+}$ pump-activity, and protein kinase C translocation.**—Paragh, G., E. Kovács, I. Seres, T. Keresztes, Z. Balogh, J. Szabó, F. Teichmann, and G. Fóris. **Altered signal pathway in granulocytes from patients with hypercholesterolemia.** ***J. Lipid Res.*** **1999.** 40: **1728–1733.**

**Supplementary key words** hypercholesterolemia • granulocytes • formyl-Met-Leu-Phe • $Ca^{2+}$ signal • inositol phosphates • leukotrienes • $O_2^-$ generation • membrane fluidity

The knowledge about the intracellular signaling of the chemotactic peptide formyl-Met-Leu-Phe receptor (FMLP-R) is almost complete. It has been demonstrated by many authors that human polymorphonuclear leukocytes (PMNLs) exhibit an FMLP-R-mediated signal transduction through a G protein phospholipase C (PLC) activation, inositol phosphate (IP) generation, $Ca^{2+}$ signaling, arachidonic acid (AA) cascade, and protein kinase C (PKC) activation (1–4).

In our previous studies we demonstated that in PMNLs obtained from healthy aged subjects and from a group of patients with non-insulin-dependent diabetes mellitus (NIDDM), the superoxide anion generation decreased whereas the intracellular killing capability remained unchanged (5, 6). In addition, in both healthy aged and NIDDM groups, the FMLP-stimulated PMNLs responded with low $IP_3$ and $Ca^{2+}$ signaling whereas the AA cascade was increased (7, 8). On the other hand, Bonneau et al. (9) found that after in vitro LDL treatment the FMLP-induced superoxide anion generation was enhanced and the signal pathway of FMLP-R was altered. In addition, low density lipoprotein (LDL) was also able to increase the oxidative burst in granulocytes. This latest result supports our previous observations that LDL induced a significant oxidative burst in human monocytes (10). The aim of the present study was to determine the signal pathway of FMLP-R in PMNLs obtained from patients with hypercholesterolemia (HCh) (high LDL / HDL ratio). These ex vivo experiments with PMNLs led to a result similar to that of the in vivo experiments of Bonneau et al. (9). The FMLP-triggered superoxide anion generation in the patient group was increased and independent of pertussis toxin (PT)-sensitive G protein whereas in $Ca^{2+}$-free medium it was inhibited. The decrease in FMLP-induced IP generation and $Ca^{2+}$ signaling and the enhanced leukotriene synthesis indicated an altered signal transduction of FMLP-R in HCh-PMNLs. Finally, the elevation of membrane-bound PKC activity, the increase in $[Ca^{2+}]i$, the decreased membrane fluidity, and the increase in cell-bound choles-

Abbreviations: AA, arachidonic acid; CaM, calmodulin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DAG, diacylglycerol; FMLP, formyl Met-Leu-Phe; HBSS, Hank's balanced salt solution; HCh, hypercholesterolemia; HDL, high density lipoprotein; IP, inositol phosphate; LDL, low density lipoprotein; LT, leukotriene; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; NIDDM, non-insulin-dependent diabetes mellitus; PA, phosphatidic acid; PBS, phosphate-buffered saline; $PGB_2$, prostaglandin $B_2$; PI, phosphatidylinositol; PKC, protein kinase C; $PLA_2$, phospholipase $A_2$; PLC, phospholipase C; PMNL, polymorphonuclear leukocyte; PT, pertussis toxin; R, receptor; V, verapamil.

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terol content in resting PMNLs of HCh patients suggest the alterations of membrane functions to be a possible consequence of the high in vivo LDL concentrations. It should be noted that in neutrophils of hypercholesterolemic patients both the enhanced membrane bound cholesterol content and the decreased membrane fluidity are well known (11, 12). On the other hand, membrane cholesterol, present almost entirely in the unesterified form, is an important modulator of cell membrane fluidity (13). In our earlier study we detected the above-mentioned membrane alterations in lymphocytes of HCh patients associated with altered immune functions (14).

## MATERIALS AND METHODS

### Patients

We examined 15 male patients suffering from hypercholesterolemia and 11 age-matched healthy male volunteers (Group 1). All patients were previously examined for LDL-R activity. Neither labeled LDL binding nor intracellular degradation was different from the controls (15). Therefore, in our patients we excluded the role of decreased LDL receptor density in the pathogenesis of HCh. In each case familial origin was also excluded. Both patients and control volunteers were kept on an NCEP Step I diet. The demographic data of investigated subjects are illustrated in **Table 1**. Exclusion criteria included fever, liver, thyroid and kidney disease, infective disorders, and antilipidemic drug use. Serum cholesterol and triglyceride were assayed with a Boehringer Mannheim GmbH Diagnostic enzyme kit, while HDL cholesterol was measured by the phospho-tungstate-magnesium precipitation method (16). The LDL cholesterol fraction was calculated indirectly using the Friedewald equation (17). Apolipoprotein examination was performed with an immuno-nephelometric assay in which the Orion Diagnostic kit was used. Venous blood samples (10–15 ml) were taken at intervals of 4–5 days from 3–5 patients and 2–3 control volunteers for each set of experiments.

The experiments were started on recently freshly diagnosed 15 male HCh patients and 11 male healthy volunteers. In view of the results obtained, the experiments were expanded to include another 27 control subjects and 34 HCh patients (Group 2) to elucidate the role of the cell-bound cholesterol content and membrane fluidity in the altered reaction to FMLP-stimulation in HCh neutrophils. The study was approved by the local ethics committee of the scientific board of the University Medical School of Debrecen.

TABLE 1. Demographic data of investigated control subjects and patients with hypercholesterolemia

| | Control Volunteers (n = 38) | | Patients with Hypercholesterolemia (n = 49) | |
|---|---|---|---|---|
| Parameters | Group 1 (n = 11) | Group 2 (n = 27) | Group 1 (n = 15) | Group 2 (n = 34) |
| Age (yr) | 56.4 ± 7.8 | 54.8 ± 8.3 | 58.6 ± 8.1 | 55.7 ± 7.6 |
| BMI (kg/m$^2$) | 22.6 ± 3.5 | 23.1 ± 3.2 | 23.8 ± 3.0 | 23.7 ± 3.6 |
| WHR (waist hip ratio) | 0.88 ± 0.12 | 0.84 ± 0.09 | 0.92 ± 0.13 | 0.90 ± 0.11 |
| Cholesterol (mm) | 4.5 ± 0.48 | 4.3 ± 0.50 | 8.4 ± 1.3 | 8.8 ± 1.1 |
| Triglyceride (mm) | 1.7 ± 0.22 | 1.9 ± 0.18 | 1.8 ± 0.24 | 1.9 ± 0.20 |
| HDL-C (mm) | 1.31 ± 0.15 | 1.38 ± 0.18 | 1.28 ± 0.14 | 1.30 ± 0.15 |
| LDL-C (mm) | 3.21 ± 0.41 | 3.83 ± 0.52 | 6.11 ± 0.72 | 6.34 ± 0.68 |

Each value represents the mean ± SD.

### Isolation of PMNLs

PMNLs were separated by Ficoll-Hypaque density gradient centrifugation according to the method of Boyum (18). The cell suspensions were 95% pure for PMNLs as judged by morphological criteria, and 96% were viable.

### Culture conditions

Cell suspensions were performed in serum-free HBSS with the appropriate cell densities. All incubations were carried out in a $CO_2$ incubator ($CO_2$: 5%, air: 95%, humidity: 95%) at 37°C. The PMNLs were stimulated with 10 nm FMLP (Serva).

### Measurement of superoxide anion generation

The superoxide anion production was measured in response to stimulation by 10 nm FMLP for 30 min, using superoxide dismutase inhibitable reduction of ferricytochrome c (Sigma) as described by Cohen and Chovaniek (19). In some of the experiments PMNLs were preincubated with 7.5 nmol/ml pertussis toxin (Calbiochem) for 120 min or with 1 μm verapamil (Sigma) for 60 min. In these latter experiments the measurements were carried out in $Ca^{2+}$-free medium containing 3 mm EGTA. Results are presented as nmol $O_2^-$ released during a 30-min incubation/$10^6$ PMNLs.

### Measurement of leukotrienes (LT)

The LT synthesis by PMNLs was determined according to the method of Jubiz, Nolan, and Kaltenborn (20) with slight modification by Huwyler and Gut (21). LT standards, namely $LTB_4$, $LTC_4$, $LTD_4$, and $LTE_4$ from Sigma (St. Louis, MO), and $PGB_2$ from Merck (Darmstadt, Germany) as the internal standard, were applied. The cells ($10^7$/ml PBS) were stimulated under constant stirring at 37°C in a $CO_2$ incubator in the presence of 10 nm FMLP. The cell suspension was spiked with a corresponding amount of internal standard $PGB_2$. After 30 min, 100 μl of cell suspension was added to 300 μl of isopropanol containing sufficient formic acid to give a final pH of 3.0. A 3-fold volume of dichloromethane mixture was added under vortexing, and phase separation was achieved by centrifugation at 10,000 rpm for 10 min. The aqueous top layer and the interphase material were discarded. Ultra-pure water (25 μl) was added to the organic phase under vortexing and the sample was centrifuged at 10,000 rpm for 2 min. The sample volume was then reduced to a minimal volume (20–30 μl) under a stream of nitrogen at about 50°C. The residue was added to 100 μl eluent and after filtration (Millipore membrane of 0.2 μm) was injected onto the HPLC, using a 20-μl loop connected to a Rheodyne 7125 valve. Determinations of leukotrienes were performed with reverse, high-phase performance liquid chromatography (HPLC, Merck–Hitachi, Darmstadt, Germany) and ultraviolet detection at a wavelength of 280 nm (UV-VIS, L-4250 detector). Separation was achieved with a LiChropher 100 RP.18 column (particle size 5 μm; 5.0 mm i.d., 125 mm long) using an iscratic elution of methanol–water 60:40, adjusted to pH 7.4 with glacial acetic acid containing 0.25% $Na_4EDTA$ (Merck, Darmstadt, Germany) at a flow rate of 1.2 ml/min. The retention time and peak heights of $PGB_2$ as internal standard were used to identify and calculate the concentration of leukotrienes in each sample. Data were processed using a model of D-6000 HPLC-Manager Software (Merck, Darmstadt, Germany).

### Measurement of inositol phosphates

The determinations were carried out according to the method of Dillon et al. (1), modified by Shayman and BeMeut (22) and Patthy, Balla, and Arányi (23). An aliquot of PMNL suspension ($10^7$ cells/ml) in HEPES-buffered HBSS was preincubated for 4 h at 37°C in the presence of 25 μm (100–200 μCi) myo-[$^3$H]inositol (Amersham) and 10 nm LiCl using a $CO_2$ incubator equipped

TABLE 2. Release of superoxide anion from PMNLs of controls and patients with HCh

| Group 1 | n | Pretreatment | Stimulation | $O_2^-$ Generation |
|---|---|---|---|---|
| | | | | *nmol/10⁶ PMNL/30 min* |
| Control | 11 | none | none | 11.1 ± 1.8 |
| | | none | 10 nm FMLP | 102.8 ± 11.4 |
| | | PT | 10 nm FMLP | 28.6 ± 3.1[b] |
| | | V + $Ca^{2+}$-free | 10 nm FMLP | 63.7 ± 7.8[b] |
| HCh | 15 | none | none | 24.8 ± 3.1[a] |
| | | none | 10 nm FMLP | 179.6 ± 19.8[a] |
| | | PT | 10 nm FMLP | 188.9 ± 20.1[a] |
| | | V + $Ca^{2+}$-free | 10 nm FMLP | 58.4 ± 8.7[a,b] |

PT: cells were pretreated with 7.5 nmol/ml pertussis toxin for 120 min; V: cells were pretreated with 1 μm verapamil for 60 min and measurement occurred in a $Ca^{2+}$-free medium containing 3 mm EDTA.

[a] Values differ from the controls significantly $P < 0.01$.

[b] The PT and $Ca^{2+}$-free medium induced inhibitions were significant, $P < 0.01$.

with a shaker. After vigorous washings, the cell-bound radioactivity was determined. The incorporated myo-[$^3$H]inositol was at least 50% of the total applied radioactivity. These cells were then stimulated with 10 nm FMLP for 20 sec. The reaction was terminated with ice-cold perchloric acid and neutralized with saturated $KHCO_3$. The precipitate was centrifuged and filtered through a 0.45-μm Milipore filter. The IPs were isolated by reverse-phase ion-pair chromatography using $IP_1$, $IP_2$, and $IP_3$ as internal standards (Amersham). After fractionation, the radioactivities were determined in a Packard 2200 CA liquid scintillation counter. The amounts of produced $IP_1$, $IP_2$, and $IP_3$ were expressed as the percentages of dpm in the corresponding resting PMNLs.

## Measurement of $[Ca^{2+}]i$

$[Ca^{2+}]i$ was determined as described by McCormach and Cobbold (24). Briefly, to 1.0 ml PMNL suspension containing $5 \times 10^6$ cells, 20 μl Indo 1/AM (Calbiochem) was added from a stock solution (1.0 nmol/L). The mixture was incubated for 30 min at 37°C in a shaker. The cells were then washed vigorously and aliquots were resuspended in HBSS. The determination of $[Ca^{2+}]i$ was carried out in a spectrofluorimeter (Hitachi F-4500) at 405 and 485 nm under constant stirring at 37°C. The final mixture consisting of $10^6$ PMNLs in 2.0 ml HBSS was placed into a cuvette, and the cells were stimulated with 10 nm FMLP during measurement. The peaks were determined after stimulation for 100–120 sec both in the control and in the HCh groups. In some experiments PMNLs were stimulated after a 60-min preincubation in 1.0 μm verapamil and the measurements were performed in $Ca^{2+}$-free HBSS consisting of 3 mm EGTA. The $[Ca^{2+}]i$ levels were calculated according to the given equation.

## Measurement of PKC activity

The method was carried out as described by Bell, Hannun, and Loomish (25) and modified by Gopalakrishna et al. (26). After a 2-min stimulation with 10 nm FMLP, the PMNL suspensions ($5 \times 10^6$ cells) were rapidly centrifuged at 4°C. The pellet was resuspended in HEPES-buffered ice-cold HBSS containing EDTA, 0.5 mmol/L EGTA, phenylmethylfluoride (Sigma), and leupeptin (Sigma). Cells were disrupted ultrasonically (Branson Sonifier 450) and centrifuged at 100,000 *g* for 45 min at 4°C (Beckman L-5-65B). Both the supernatants containing the cytosol and the pellets were then solubilized with CHAPS (Sigma) and 1% Nonidet P-40 (Sigma). The pellets were rehomogenized and centrifuged again as above. The PKC activities of cytosolic and membrane fractions were determined by measuring the $^{32}$P incorporation from [$^{32}$P]ATP (Institute of Radiochemical Research, Budapest) into 100 μg/ml histone III-S (Sigma) in the presence of 10 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$, 96 μg/ml l-phosphatidyl-l-serine (Sigma), 6.5 μg/ml oleoyl-2-acetyl-*sn*-glycerol (Sigma), 50 μmol/L adenosine triphosphate $Na_2ATP$ (Sigma) and 100–200 cpm/mg [$^{32}$P]ATP. The reaction was terminated after 10 min by adding ice-cold trichloroacetic acid and bovine serum albumin as carrier. The precipitate was then filtered through a 0.45-μm Milipore HA filter and washed in 5 × 2 ml ice-cold trichloroacetic acid. The radioactivity was determined with a Packard 2200 CA liquid scintillation counter using a toluol cocktail to dissolve the filter. The PKC activity was expressed as incorporated $^{32}$P pmol/min per mg protein.

TABLE 4. Effect of 10 nm FMLP on the increase in inositol phosphate production in PMNLs obtained from control subjects and patients with HCh

| Group 1 | n | $IP_1$ | $IP_2$ | $IP_3$ |
|---|---|---|---|---|
| | | % | % | % |
| Control | 11 | 161.8 ± 20.4 | 132.3 ± 15.8 | 198.5 ± 24.7 |
| HCh | 15 | 114.2 ± 11.9[a] | 105.4 ± 11.8[a] | 122.3 ± 14.7[a] |

Each value represents the mean ± SD.

[a] The decrease in $IP_1$, $IP_2$, and $IP_3$ production in the HCh group was statistically significant, $P < 0.01$.

## Measurement of cell cholesterol

Cholesterol content was measured by the method of Goh, Krauth, and Colles (27). Neutrophils ($10^7$ cells/ml) were digested in a solution containing 0.1% sodium dodecyl sulfate (Sigma), 1 mm EDTA (Sigma), and 0.1 m Tris buffer, pH 7.4, at 30°C for 5 min. The gelatinous mixture was homogenized by discharging it 5 times through a 21-gauge needle attached to a 3-ml syringe. To a 400-μl aliquot of suitably diluted cell digest was added 100 μl of a mixture containing 150 mm sodium phosphate, pH 7, 30 mm sodium taurocholate, 1.02 mm polyethylene glycol (mol. wt. 8000) and 0.2 units cholesterol oxidase (Sigma), 0.4 units of horseradish peroxidase type IV (Sigma), 0.4 mg P-hydroxyphenylacetic acid. The mixture was incubated for 60 min at 30°C. Two milliliters of 50 mm sodium phosphate, pH 7.4, was added after the incubation, and sample fluorescence was determined at an excitation wavelength of 415 nm and an emission wavelength of 415 nm using a Hitachi F-4500 spectrofluorimeter.

TABLE 3. FMLP-induced leukotriene synthesis in PMNLs obtained from control subjects and patients with HCh

| Group 1 | n | $LTB_4$ | $LTC_4$ | $LTD_4$ | $LTE_4$ |
|---|---|---|---|---|---|
| | | | *ng/10⁷ cells* | | |
| Control | 11 | 19.753 ± 3.11 | 10.324 ± 1.18 | 16.337 ± 1.88 | 2.753 ± 0.11 |
| HCh | 15 | 38.965 ± 4.12[a] | 36.645 ± 4.35[a] | 28.673 ± 2.55[a] | 18.783 ± 3.01[a] |

Results are expressed as means ± SD.

[a] Differences from control group are significant, $P < 0.01$.

TABLE 5. Protein kinase C activity in resting PMNLs obtained from controls and patients with HCh

| Group 1 | n | Cytosolic Fraction | Particulate Fraction | Total Activity |
|---|---|---|---|---|
| Control | 11 | 2122 ± 198 | 237 ± 22 | 2298 ± 236 |
| HCh | 15 | 2301 ± 202 | 1688 ± 182[a] | 3657 ± 402[a] |

Protein kinase C activity was expressed as pmol $^{32}P$/min/mg protein. Each value represents the mean ± SD.

[a] Values in the HCh group differ from the control values significantly, $P < 0.01$.

TABLE 7. Cell-bound cholesterol content in PMNLs obtained from healthy controls and patients with HCh

| Group 2 | n | Cell-Bound Cholesterol |
|---|---|---|
| | | *μg/10⁷ cells* |
| Control | 27 | 4.693 ± 0.455 |
| HCh | 34 | 9.732 ± 1.287[a] |

[a] Values between control and HCh group differ significantly, $P < 0.01$.

### Determination of membrane fluidity

The membrane fluidity was measured according to the method of Shinitzky and Yuli (28) by fluorescence polarization using DPH. The DPH dispersion (2.15 μmol/l) was mixed with $5 \times 10^6$ cell/ml PBS in a 1:1 proportion, followed by a 30-min incubation in the dark. After washing, cells were analyzed for fluorescence polarization in a spectrofluorimeter (Hitachi F-4500) equipped with a polarization attachment and thermostatic cell holder. The excitation was carried out at 355 nm and the emission at 430 nm was determined. The polarization value (P) obtained is inversely proportional to the fluidity of the cell membrane.

### Statistics

The statistical significance of the results was calculated by Student's *t*-test. The interassay coefficient did not exceed 15%.

## EXPERIMENTAL RESULTS

Data in **Table 2** show that the FMLP-triggered oxidative burst was significantly higher in the patient group than in the controls. In the control cells, PT markedly inhibited the FMLP triggered $O_2^-$ generation. In turn, in the HCh group, the G protein inhibitory PT did not affect the increased superoxide anion release. However, when experiments were carried out after pretreatment with verapamil and the measurement of $O_2^-$ occurred in $Ca^{2+}$-free buffer, the FMLP-triggered oxidative burst was inhibited more intensively in the HCh-PMNLs than in the control cells. The next experiments with the FMLP-induced LT synthesis in the PMNLs of control and HCh patients are demonstrated in **Table 3**. Data show a significantly increased synthesis of $LTB_4$, $LTC_4$, $LTD_4$, and $LTE_4$ in HCh-PMNLs. To identify the signal transduction of FMLP-R in HCh-PMNLs, the $IP_1$, $IP_2$, and $IP_3$ generations were measured after FMLP stimulation (**Table 4**). According to our results, FMLP caused a significant increase in IP production in control cells whereas it failed to induce IP synthesis in PMNLs of the HCh group.

**Table 5** demonstrates that resting PMNLs from HCh patients have nearly twice as much $[Ca^{2+}]i$ as resting PMNLs from the control groups. The FMLP-induced $Ca^{2+}$ signaling is increased significantly and the time to normalization appears to be prolonged in PMNLs obtained from the HCh patients. In HCh-PMNLs, the $Ca^{2+}$ peak significantly decreased after verapamil pretreatment and in $Ca^{2+}$-free medium. In control cells, the $Ca^{2+}$ signal is independent of both the extracellular $Ca^{2+}$ and $Ca^{2+}$ channels. This is indicated by the absence of a significant decrease in the $Ca^{2+}$ peak measured in $Ca^{2+}$-free medium after incubation with 1.0 μm $Ca^{2+}$ channel blocking verapamil. The PT-resistant increase in oxidative burst and the intensive LT synthesis associated with the failure of $IP_3$ and $Ca^{2+}$ signaling from intracellular pools, as well as the $Ca^{2+}$ influx from the medium, suggest the significance of an alternative signal pathway through $PLA_2$ activation and arachidonic acid cascade. On the other hand, the elevated $[Ca^{2+}]i$ in resting HCh-PMNLs and the delayed normalization of the FMLP-triggered $Ca^{2+}$ signal also indicate the potential role of membrane alteration in resting PMNLs of patients with HCh. In resting PMNLs from patients with HCh, the membrane-bound and consequently the total PKC activity increased significantly compared to the values of control PMNLs (**Table 6**). Finally, the cell-bound cholesterol content was measured in PMNLs obtained from controls and HCh patients (**Table 7**). Data show the increase in cholesterol

TABLE 6. Cytosolic free $Ca^{2+}$ level, the $Ca^{2+}$ signal and its time-dependency in FMLP-stimulated PMNLs of controls and patients with HCh

| Group 1 | n | Treatment | $[Ca^{2+}]i$ Basal Level | $\Delta[Ca^{2+}]i$ $Ca^{2+}$ Signal | Time to Return |
|---|---|---|---|---|---|
| Control | 11 | HBSS | 158.5 ± 14.9 | 101.6 ± 12.4 | 123.7 ± 14.5 |
| | | $Ca^{2+}$-free medium + V | 136.1 ± 14.2 | 90.4 ± 8.7 | 133.1 ± 14.3 |
| HCh | 15 | HBSS | 253.3 ± 31.0[a] | 165.1 ± 15.3[a] | 289.9 ± 35.7[a] |
| | | $Ca^{2+}$-free medium + V | 264.8 ± 29.9[a] | 28.5 ± 2.5[b] | 322.0 ± 34.7[a] |

Each value represents the mean ± SD. The $[Ca^{2+}]i$ was calculated as nmol/L. The $Ca^{2+}$ peak ($\Delta Ca^{2+}$) was determined at the 2nd min after stimulation. Cells were preincubated with 1.0 μm verapamil (V) (Serva) for 60 min.

[a] Values differ significantly from control values, $P < 0.01$.

[b] The $Ca^{2+}$ peak ($\Delta[Ca^{2+}]i$) decreased significantly after verapamil pretreatment and in $Ca^{2+}$-free medium, $P < 0.01$.

TABLE 8. Values of fluorescence polarization (P) of DPH in PMNLs of control subjects and patients with HCh

| Group 2 | n | P |
|---|---|---|
| Controls | 27 | 0.396 ± 0.005 |
| HCh | 34 | 0.504 ± 0.007[a] |

Results are expressed as means ± SD.
[a] Differences between controls and patients with HCh are significant, $P < 0.001$.

content in the patient group compared to the controls. The membrane fluidity was determined both in control and HCh groups (**Table 8**), to elucidate the given alterations in signaling and basal metabolism in PMNLs obtained from patients with high LDL levels. The P polarization value was significantly higher in the patient group than in the controls, suggesting a decreased membrane fluidity in PMNLs of HCh patients.

## DISCUSSION

FMLP, as one of many chemotactic peptides, activates NADPH oxidase through the PI cleavage, DAG, PA, and AA pathway (29–32). Our results suggest that in PMNLs of HCh patients, FMLP does not affect cells through PLC activation, $IP_3$ production, and $Ca^{2+}$ signaling from intracellular pools. Rather, the signal transduction occurs through $Ca^{2+}$ influx, $PLA_2$ activation, and AA cascade; and this alternative pathway (33) is responsible for the enhanced superoxide anion generation and LT synthesis in HCh-PMNLs (34). Consequently, the increased $Ca^{2+}$ influx inducing the $PLA_2$ activation followed by an intensive AA cascade can lead to superoxide generation and PKC activation and translocation (2, 4, 29, 35, 36). Data in Table 2 suggest that the sensitivity of $O_2^-$ generation was more intensive for extracellular $Ca^{2+}$ in the HCh group than in control cells. The role of superoxide anion and LTs in the pathogenesis of atherosclerosis is well known, acting either by LDL oxidization (35) or by endothelial injury (38–40). Therefore, we assume that PMNLs of patients with HCh play a significant role in the pathogenesis of atherosclerosis without any remarkable impairment of nonspecific resistance against pathogens. The high $[Ca^{2+}]i$ in resting PMNLs of HCh patients, and in particular the delayed normalization of the $Ca^{2+}$ level after $Ca^{2+}$ influx, suggest an altered function of $Ca^{2+}$ pumps responsible for $Ca^{2+}$ efflux from cells. The delayed normalization of $[Ca^{2+}]i$ after FMLP stimulation may be a consequence of the impairment of calmodulin (CaM)-dependent membrane $Ca^{2+}$ pumps (41, 42). Another question concerns the high activity of membrane-bound PKC activity in the resting HCh-PMNLs: it may not exclude the association between the impaired function of CaM-dependent $Ca^{2+}$ pumps and altered membrane-bound PKC activity, and both may be caused by the increased amount of cell-bound cholesterol and decreased membrane fluidity (43).

Finally, based on these ex vivo experiments, it is assumed that the high LDL level in the circulation of patients with HCh affects the PMNL–membrane composition, fluidity, and consequently a number of membrane-bound cell functions including those of $Ca^{2+}$ channels, $Ca^{2+}$ pumps, and PKC activity.

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