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Angiotensin II–induced oxidative burst is fluvastatin sensitive in neutrophils of patients with hypercholesterolemia

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Abstract

The aim of this study was to investigate the effect of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor fluvastatin (Flu) on angiotensin II (AII)–stimulated neutrophils of patients with hypercholesterolemia. Results suggest that a 6-week-long Flu administration completely counteracted the AII-induced increase in superoxide anion and leukotriene C_4 production of the neutrophils of patients with hypercholesterolemia. However, the failure of signal processing through pertussis toxin–sensitive G protein, the increase in $[Ca^{2+}]_i$ in membrane-bound protein kinase C activity, and the increase in neutrophil-bound cholesterol content were only partially restored by Flu. In addition, Flu had no effect on the increased membrane rigidity of the neutrophils of patients with hypercholesterolemia. To sum it up, Flu administration had a beneficial effect on AII-triggered reactive oxygen species generation; it resulted in partial restoration of signaling processes and of membrane composition, but membrane fluidity remained unchanged.

1. Introduction

In our previous publications, we described that angiotensin II (AII) could activate human neutrophils of control subjects and patients with hypercholesterolemia (HC) through different intracellular signaling pathways. The AII-induced superoxide anion and leukotriene C_4 (LTC_4) generation was significantly higher in neutrophils of patients with HC than in those of healthy subjects [1]. In this study, we investigated the possible beneficial effect of fluvastatin (Flu) on AII-stimulated neutrophils of healthy volunteers. One of the effects of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor statins against atherosclerosis is their antioxidant feature. Various statins are able to inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to a different extent through the inhibition of the isoprenoid pathway [2]. The NADPH oxidase, which is the key enzyme of superoxide anion production in neutrophils, is regulated by Rac2 guanosine triphosphatase (GTPase) from the Rho family, which is activated by the mevalonate cycle through geranylgeranylation [3-5]. At the same time, AII plays a role in the pathomechanism of atherosclerosis, not only as a hypertension-inducing neurohormone but also through its oxidative burst–triggering ability [6-9]. In addition, on the site of plaque formation, the local production of AII is increased and AII type 1 (AT1) receptors are up-regulated [10].

Furthermore, our studies have shown that in human neutrophils of individuals with hyperlipidemia, the chemotactic peptide formyl-Met-Leu-Phe and AII were unable to induce either $Ins(1,4,5)P_3$ (IP_3) generation or Ca^{2+} signals from intracellular pools despite the intensive production of superoxide anion and leukotrienes [1,11].

The goals of the present study were (1) to determine the possible beneficial effect of a 6-week-long Flu treatment on the pathological release of oxidizing agents by neutrophils of patients with HC and (2) to evaluate whether Flu administration is able to restore the disturbed signaling pathway and membrane status.

2. Methods

2.1. Patients

We enrolled 28 male patients with type IIa HC who were not to receive any treatment, 21 male patients with HC

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Table 1
Demographic data of investigated control subjects and Flu-treated and nontreated patients with HC

Parameters	Control subjects (n = 34)	Patients with HC (n = 28)	Patients with HC + Flu[a] (n = 21)
Age (y)	58.2 ± 6.3	55.7 ± 6.0	59.2 ± 6.87
Body mass index (kg/m^2)	23.8 ± 3.2	22.6 ± 3.5	24.1 ± 3.8
WHR[b]	0.86 ± 0.11	0.95 ± 0.15	0.92 ± 0.13
Cholesterol (mmol/L)	4.6 ± 0.51	8.7 ± 1.0	5.9 ± 0.68[c]
Triglyceride (mmol/L)	1.7 ± 0.22	1.9 ± 0.24	1.7 ± 0.20
HDL cholesterol (mmol/L)	1.35 ± 0.15	1.31 ± 0.15	1.40 ± 0.16
LDL cholesterol (mmol/L)	3.43 ± 0.41	6.72 ± 0.78	4.11 ± 0.55[c]

Each value represents the mean ± SD.

[a] The following data represent Flu administration's favorable results after 6 weeks: decrease in cholesterol, 17.4; in LDL-C, 26.6; in apolipoprotein B100, 18.3; and in LDL/HDL, 28.1%.

[b] Waist hip ratio.

[c] Differences between Flu-treated and nontreated groups of patients are significant ($P < .01$).

treated for 6 weeks with 40 mg/d of Flu (Lescol, Novartis, Budapest, Hungary), and 34 age-matched healthy male volunteers. In the patients with HC, both low-density lipoprotein (LDL) receptor failure and familial origin were excluded. Patients as well as control volunteers were kept on a National Cholesterol Education Program step I diet. The demographic data of the investigated subjects are summarized in Table 1. The effect of Flu was not studied on the same group of patients with HC (ie, the experiment was not self-controlled). In the Flu-treated HC group, the changes in lipid parameters were as follows: plasma total cholesterol, −17.4%; high-density lipoprotein (HDL) cholesterol, +5.9%; triglycerides, −9.6%; apolipoprotein A1, +6.2%; apolipoprotein B100, −18.3%; LDL/HDL, −28.1%; LDL cholesterol, −24.6%. Exclusion criteria were liver, thyroid, and kidney diseases; diabetes mellitus; infective disorders; fever; and lipid-lowering medication use. A Cobas Integra 700 Analyzer (Roche, Basel, Switzerland) was used for lipid measurements. The LDL cholesterol fraction was calculated indirectly using the Friedewald equation. Apolipoprotein examination was performed with an immunonephelometric assay in which an Orion Diagnostic kit (Orion Diagnostica, Espoo, Finland) was used. Venous blood samples (10-15 mL) were taken from healthy volunteers and patients at intervals of 4 to 5 days for each set of experiment. All determinations were carried out parallel in each patient group instead of using a self-controlled system. The study protocol was approved by the local ethics committee of the Medical and Health Science Center, University of Debrecen, and informed consent was obtained from all patients.

2.2. Isolation of neutrophils

Neutrophils were separated by Ficoll-Hypaque density gradient centrifugation according to the method of Boyum [12]. The cell suspensions were 95% pure for neutrophils as judged by morphological criteria, and 96% were viable.

2.3. Culture conditions

Cell suspensions were performed in a serum-free Hanks balanced salt solution (HBSS) with the appropriate cell densities. All incubations were carried out in an ASSAB CO_2 incubator (humidity, 95%; air, 95%; CO_2, 5%) at 37°C. For in vitro stimulation of neutrophils, AII (Serva, Heidelberg, Germany) was used, and as we described previously, the appropriate immunostimulating concentration was determined by the intracellular killing measurement [1,8,9]. Based on these experiments, 10 nM of AII was the most effective concentration to stimulate human neutrophils. In some experiments, neutrophils at 10^6/mL density were preincubated with different inhibitory drugs before AII stimulation. These were 7.5 nmol/mL of pertussis toxin (PT) (Calbiochem, La Jolla, Calif) for 120 minutes, 1.0 μM of Ca^{2+} channel-blocking verapamil (V, Sigma Co, St Louis, Mo) for 60 minutes, and 1.0 μM of phospholipase A_2 (PLA_2) inhibitory mepacrin (M, Sigma) for 60 minutes. We applied the above concentrations based on our previous studies and, in part, on the literature [13-17].

2.4. Superoxide anion generation

The O_2^- was measured as we previously described in detail [18]. To briefly sum it up, the determination was carried out spectrophotometrically by measuring the reduction of cytochrome C (type IV, Sigma), according to the method of Babior et al [19], in a microassay using a 96-well microplate and an ELISA reader (Anthos Labtec, Wien, Austria). Neutrophils were stimulated with AII at a final concentration of 10 nM. Each experiment was performed in triplicate. Results were expressed as nanomoles of produced O_2^- per 30 minutes per 10^6 neutrophils.

2.5. Measurement of LTC_4

The leukotriene synthesis of neutrophils was determined according to the method of Jubiz et al [20] with slight modifications. Leukotriene C_4 (Sigma) was applied as internal standard. After the preincubation of the neutrophils (at 10^7 polymorphonuclear leukocytes/mL density) with different inhibitory drugs as described above, they were stimulated under constant stirring with 10 nM of AII in a CO_2 incubator at 37°C. As in our earlier experiments, LTC_4 production was the most pronounced among all leukotrienes triggered by AII. Therefore, only LTC_4 determination was performed (at 280 nm), as described previously [10]. Data were processed using a model of D-6000 HPLC Manager Software (Merck, Darmstadt, Germany).

2.6. Determination of IP_3

Determination of IP_3 was carried out according to the method of Shaymann and BeMeut [21]. An aliquot of neutrophil suspension (10^7 cells/mL) in HEPES-buffered HBSS was incubated for 4 hours at 37°C in the presence of 100 to 200 μCi of *myo*-[^{3}H]inositol (Amersham, Braunschweig, Germany) and 10 nmol/L of LiCl using a CO_2

incubator equipped with a shaker. The appropriate concentrations of inhibitory drugs were added to the aliquots of this mixture 10 to 120 minutes before the completion of preincubations with *myo*-[^{3}H]inositol. After vigorous washing, cell-bound radioactivity was determined. The incorporated *myo*-[^{3}H]inositol was at least 50% of the total applied radioactivity even in the presence of inhibitory drugs in the samples. Neutrophils were stimulated with 10 nM of AII, and after 2 minutes, the reaction was terminated with ice-cold perchloric acid and neutralized with saturated $KHCO_3$. Ins(1,4,5)P_3 was isolated by reversed-phase ion-pair chromatography using IP_3 as internal standard (Amersham). The amount of produced IP_3 was expressed as a percentage of disintegrations per minute to the corresponding disintegrations per minute of resting neutrophils.

2.7. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined as described by McCormach and Cobbold [22] by using Indo 1/AM (Calbiochem) as described earlier [1,11]. The determination of $[Ca^{2+}]_i$ was carried out in a spectrofluorometer (Hitachi F-4500, Hitachi, Ltd, Tokyo, Japan) at 405 and 485 nm under constant stirring at 37°C. Neutrophils were stimulated with 10 nM of the final concentration of AII during measurements. In some experiments, neutrophils were preincubated with inhibitory drugs before the addition of 20 nmol of Indo 1/AM to 10^7 cells. The resultant dye intake was not altered by the preincubations. The peak of $[Ca^{2+}]_i$ increase occurred after stimulation for 100 to 200 seconds in all experimental groups. The $[Ca^{2+}]_i$ was calculated according to the indicated equation. The Ca^{2+} signals were expressed as peak $[Ca^{2+}]_i$ minus the $[Ca^{2+}]_i$ of nonstimulated resting neutrophils in nanomolars of Ca^{2+}.

2.8. Measurement of protein kinase C activity

The measurement was carried out via the method of Bell et al [23] modified by Gopalakrishna et al [24]. The freshly isolated neutrophils' suspension (5×10^6 cells) was centrifuged at 4°C. The pellets were resuspended in HEPES-buffered ice-cold HBSS containing EDTA, 0.5 mmol/L of EGTA, phenylmethyl-sulphonylfluoride (Sigma), and leupeptin (Sigma). Cells were disrupted ultrasonically (Branson Sonifier 450, Branson Ultrasonic Corp, Danbury, Conn) and centrifuged at 100 000 g for 45 minutes at 4°C (Beckman L-5-65B, Palo Alto, Calif). Both the supernatants containing cytosol and the pellets were then solubilized with CHAPS (Sigma) and 1% Nonidet P-40 (Sigma). The pellets were rehomogenized and centrifuged again as mentioned above. The protein kinase C (PKC) activities of cytosolic and membrane fractions were determined by measuring the ^{32}P incorporation from 100 to 200 cpm/mg gamma [^{32}P]-ATP (Institute of Radiochemical Research, Budapest, Hungary) into 100 μg/mL histone III-S (Sigma). The reaction was terminated after 10 minutes by adding ice-cold trichloroacetic acid and bovine serum albumin as a carrier. The precipitate was then filtered through a 0.45-μm Millipore HA filter (Millipore, Bedford, Mass) and washed in 5 × 2 mL ice-cold trichloroacetic acid. The radioactivity was determined with a Packard 2200 CA liquid scintillation counter (Packard, Meriden, Conn), using a toluol cocktail to dissolve the filter. The PKC activity was expressed as incorporated ^{32}P picomoles per minute per milligram of protein.

2.9. Measurement of cell cholesterol

Cholesterol content was measured by the method of Goh et al [25]. Neutrophils (10^7 cells/mL) were digested in a solution containing 0.1% sodium dodecyl sulfate (Sigma), 1 mmol/L EDTA (Sigma), and 0.1 mol/L Tris buffer, pH 7.4, at 30°C for 5 minutes. The gelatinous mixture was homogenized by discharging it 5 times through a 21-G needle attached to a 3-mL syringe. To a 400-μl aliquot of appropriately diluted cell digest, a 100-μl mixture containing 150 mmol/L of sodium phosphate, pH 7, 30 mmol/L of sodium taurocholate, 1.02 mmol/L of polyethylene glycol (molecular weight, 8000), 0.2 U of cholesterol oxidase (Sigma), 0.4 U of horseradish peroxidase type IV (Sigma), and 0.4 mg of *P*-hydroxyphenylacetic acid was added. The mixture was incubated for 60 minutes at 30°C. Two milliliters of 50-mmol/L sodium phosphate, pH 7.4, was added after the incubation period. Sample fluorescence was determined at an excitation wavelength of 360 nm and an emission wavelength of 405 nm using a Hitachi F-4500 spectrofluorometer.

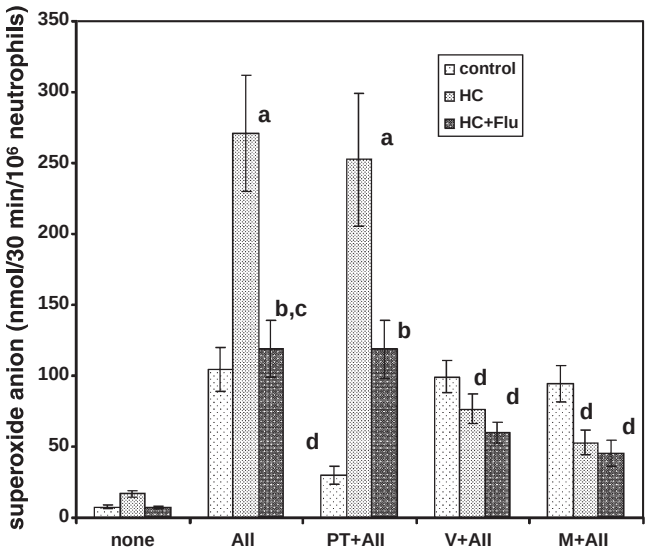

Fig. 1. The inhibition of AII-triggered superoxide anion generation of neutrophils obtained from control subjects and from nontreated and Flu-treated patients with HC. Each value represents the mean ± SD. (a) Differences of the AII-triggered increase in superoxide anion production between the control and HC groups are significant ($P < .001$). (b) Inhibition of AII-induced superoxide anion production in the HC group after Flu treatment is significant ($P < .001$). (c) Difference between the control and HC + Flu–treated groups is not significant ($P = .3293$). (d) Inhibition by in vitro preincubation is significant ($P < .001$).

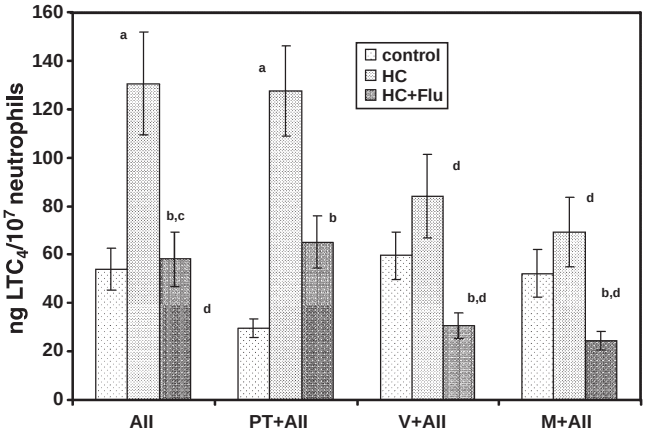

Fig. 2. Inhibition of AII–triggered LTC_4 production of neutrophils obtained from control subjects and from nontreated and Flu-treated patients with HC. Each value represents the mean ± SD. (a) Differences of AII-triggered increase in LTC_4 production between the control and HC groups are significant ($P < .001$). (b) Inhibition of AII-induced LTC_4 production in the HC group after Flu treatment is significant ($P < .001$). (c) Difference between the control and HC + Flu–treated groups is not significant ($P = .4895$). (d) Inhibition by in vitro preincubation is significant ($P < .001$).

2.10. Determination of membrane fluidity

The membrane fluidity was measured by fluorescence polarization, according to the method of Shinitzky and Yuli [26], using dimethylhexatriene (Sigma). The diphenylhexatriene dispersion (2.15 μmol/L) was mixed with 5×10^6 cell/mL of HBSS in a 1:1 proportion, followed by a 30-minute incubation in the dark. After washing the cells, they were analyzed for fluorescence polarization in a spectrofluorometer (Hitachi F-4500) equipped with a polarization attachment and thermostatic cell holder. The extinction was carried out at 355 nm and the emission at 430 nm was determined. The obtained polarization value (P) is inversely proportional to the fluidity of the cell membrane.

2.11. Statistical analysis

For mathematical analysis, paired and unpaired *t* tests with Welch's correction, analysis of variance, the Student-Newman-Keuls (SAS 6.12 program) test, and the GraphPad Software Prism Version 2.0 One-Side Binding Hyperbola (GraphPad Software, San Diego, Calif) program were applied.

3. Experimental results

Fig. 1 shows that AII induced a significantly higher O_2^- production in neutrophils of patients with HC than in those of control volunteers. However, Flu treatment abolished this enhanced oxidative burst. In neutrophils obtained from healthy subjects, the AII-triggered superoxide production was inhibited by pretreatment with G protein–inhibiting PT, but inhibition of Ca^{2+} channel opening and PLA_2 activity by V and M preincubations appeared to be ineffective. In contrast, V and M inhibited the AII-triggered enhanced superoxide release of neutrophils of patients with HC successfully. In the patient group, the AII-induced superoxide production was normalized after Flu administration, but superoxide production could be inhibited similarly to the nontreated HC group by V and M. These results suggest that Flu is not able to restore the altered signaling pathways of AII stimulation in neutrophils of patients with HC. Furthermore, an intensive AII-triggered LTC_4 production was measured in the control and patient groups, and the LTC_4 synthesis was more pronounced in the latter cases. The increase in AII-triggered LTC_4 production was completely counteracted by Flu treatment (Fig. 2). Our results obtained after preincubation with different inhibitory drugs seemed to be similar to those obtained in the case of superoxide anion production. In neutrophils of control subjects, the AII-triggered LTC_4 production was inhibited by in vitro pretreatment with PT, whereas in neutrophils of patients with HC—both in the nontreated and Flu-treated groups—the intensive LTC_4 production was inhibited by V and M. Based on these results, the beneficial effect of Flu administration on the oxidative processes of the neutrophils of patients with HC does not seem to affect the signaling pathways. To clarify this issue, the direct effect of Flu administration on AII-stimulated IP_3 elevation was assessed (Table 2). The IP_3 signaling in neutrophils of control subjects was PT sensitive, whereas AII induced a complete failure of IP_3 signaling in the cells of the HC group. The failed IP_3 signal in the HC group was in part restored by Flu administration, and this IP_3 signal appeared to be PT resistant. The increase in $[Ca^{2+}]_i$ (ie, the Ca^{2+} signals in neutrophils obtained from control subjects and patients with HC) with and without Flu administration is shown in Fig. 3. It should be noted that in resting cells obtained from patients with HC, the $[Ca^{2+}]_i$ level was significantly increased

Table 2
Angiotensin II–induced elevation of IP_3 production and its inhibition in neutrophils obtained from control subjects and from Flu-treated and nontreated patients with HC

Incubations	Control subjects		Patients with HC		Patients with HC + Flu	
	Values ± SD	*P*	Values ± SD	*P*	Values ± SD	*P*
None	100.0 ± 18.2		112.2 ± 18		103.9 ± 25.5	
AII	221.5 ± 50.1[a]	<.001	118.6 ± 14.8	.3755	175.7 ± 29.5[a]	<.001
PT + AII	125.1 ± 40.2[b]	<.001	117.0 ± 13.0	.4974	169.9 ± 33.2[b]	.6613

Values were calculated as percentage of IP_3 in nonstimulated neutrophils of control subjects.
[a] Differences between the nonstimulated (none) and AII-stimulated groups.
[b] Differences between the AII-stimulated and PT-inhibited groups.

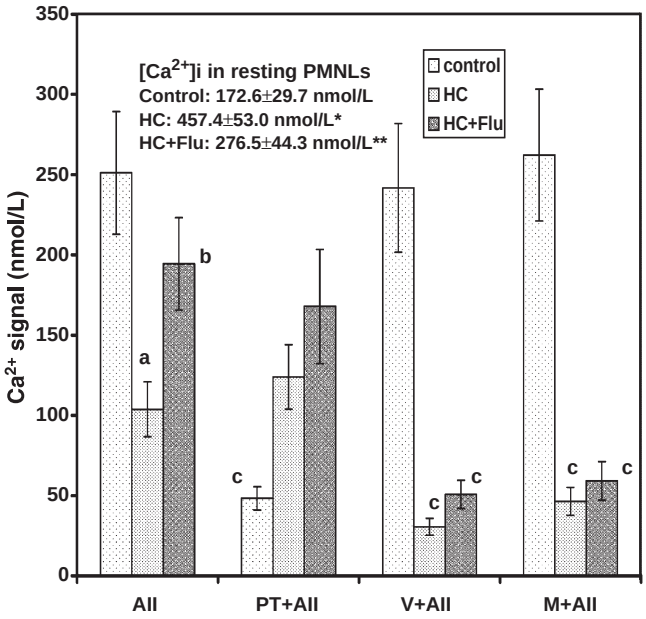

Fig. 3. Inhibition of AII-induced increase in δ-$[Ca^{2+}]_i$ (ie, Ca^{2+} signal of neutrophils obtained from control subjects and nontreated and Flu-treated patients with HC). Each value represents the mean ± SD. (a) Decrease in AII-induced Ca^{2+} signal between the control and HC groups is significant ($P < .001$). (b) Restoration of AII-induced Ca^{2+} signal in the Flu-treated group is significant ($P < .001$). (c) Inhibition of Ca signal by in vitro preincubation is significant ($P < .001$). The single asterisk indicates that differences are significant between the values in the control and HC groups ($P < .001$); double asterisk, values differ significantly from the values in the control and HC groups ($P < .001$).

compared with control subjects, and Flu was only partially able to restore the normal level of $[Ca^{2+}]_i$. The Ca^{2+} signal elicited by AII in neutrophils of patients with HC was lower than that in those of control subjects, but Flu treatment counteracted this decrease in the intensity of the Ca^{2+} signal ($P < .001$). The Ca^{2+} signal in neutrophils of control subjects triggered by AII was only inhibited by pretreatment with PT. In neutrophils of patients with HC, the signal was inhibited after in vitro treatment with V and M. The Ca^{2+} signal was inhibited in the HC + Flu group not only by V and M but also partially by PT pretreatment ($P = .0356$). Furthermore, the action of another key enzyme of signal processing—the PKC activity—was also studied in resting neutrophils and showed that the membrane-bound PKC activity increased in the untreated and Flu-treated HC groups (Table 3). Although Flu treatment was able to significantly ($P < .001$) decrease the membrane-bound PKC activity, the enzyme activity did not return to normal levels. The cell-bound cholesterol content and membrane fluidity were also measured in resting neutrophils. The cell-bound cholesterol increased significantly in neutrophils of nontreated patients with HC, whereas administration of Flu resulted in partial restoration of cholesterol in the neutrophils' membranes. Despite the fact that Flu induced a decrease in serum and cell-bound cholesterol content, the increased membrane rigidity in the HC group was not restored by Flu administration.

4. Discussion

The practical importance of the present study was to see whether AII's atherosclerosis promoting effect could be counteracted by statin administration. Angiotensin II can stimulate reactive oxygen species and has an inhibiting effect on the scavenging system. The amount of locally produced AII at the site of injured intima is uncertain for the following reasons: (1) AII is produced by systems that are independent from the renin-angiotensin system at the site of injury and the locally generated AII induces an up-regulation of AT1 receptors [27-29] and (2) AII has a concentration-dependent biphasic effect on neutrophils, and—based on our earlier studies—the neutrophils of patients with HC react more intensively to AII stimulation than those of control subjects [1].

According to our recent results, Flu counteracts the AII-induced enhanced production of superoxide anion and LTC_4 of neutrophils of patients with HC. This benefit of Flu administration may occur through its inhibitory effect on the activation of the Rac2 GTPase, which is a member of the Rho family and is involved in both NADPH oxidase and PLA_2 activation [30,31]. Regarding the reason of the absent IP_3 signal in neutrophils of patients with HC (Table 2), we may not exclude the possibility of HC-induced alterations of other kinases. These kinases, such as phosphatidylinoside-3-kinase (PI3K), phosphatidylinoside-4-kinase, and MAP kinase, are involved in the regulation of G protein–binding receptor signaling, which differs from the so-called canonical PI pathway [32-34]. It should be pointed out that our knowledge on the signaling of G protein–binding receptors

Table 3
Effect of Flu treatment on PKC activity, cell-bound cholesterol content, and fluorescence polarization (P) of diphenylhexatriene in neutrophils obtained from control subjects and nontreated and Flu-treated patients with HC

Parameters	Control subjects	Patients with HC	Patients with HC + Flu
Membrane-bound PKC[a]	243.5 ± 41.3	2089 ± 482.5[b]	683.4 ± 199.2[c]
Cell-bound cholesterol ($\mu g/10^7$ cells)	4.579 ± 0.835	9.658 ± 2.136[b]	6.273 ± 1.865[c]
Fluorescence (polarization of dimethylhexatriene)	0.3998 ± 0.011	0.6115 ± 0.0673[b]	0.6811 ± 0.1279[d]

Each value represents mean ± SD.
[a] Protein kinase C activity is expressed as incorporated ^{32}P per minute per milligram of protein.
[b] Differences between the control and HC groups are significant ($P < .001$).
[c] Differences between the control and HC + Flu groups and between HC and HC + Flu groups are significant ($P < .001$).
[d] Differences between the control and HC + Flu groups are significant, whereas between the HC and HC + Flu groups, the P value was .4099.

under pathological circumstances is incomplete, particularly in the case of AT1 receptors [35-37]. The partial restoration of IP_3 signals after Flu administration occurred through a PT-resistant pathway, whereas in the Flu-treated group, the AII-triggered Ca^{2+} signal originated through a mixed pathway either from intracellular pools or from the extracellular medium (Fig. 3). The increased amount of membrane-bound PKC in resting neutrophils of patients with HC may be the consequence of new PKC isoforms, which appear in the cell membrane. In normal human neutrophils, the ϵ, α, and β isoforms are present, whereas PKCϵ, PKCζ, and PKCδ may also appear in the case of altered membrane functions [38].

As we described earlier, AII activates the Gi protein and phospholipase C (PLC), which can be inhibited with PT, through its AT1 receptors in neutrophils of control subjects. The produced IP_3 triggers Ca^{2+} signal from intracellular pools, whereas diacylglycerol activates PLA_2 and leukotriene

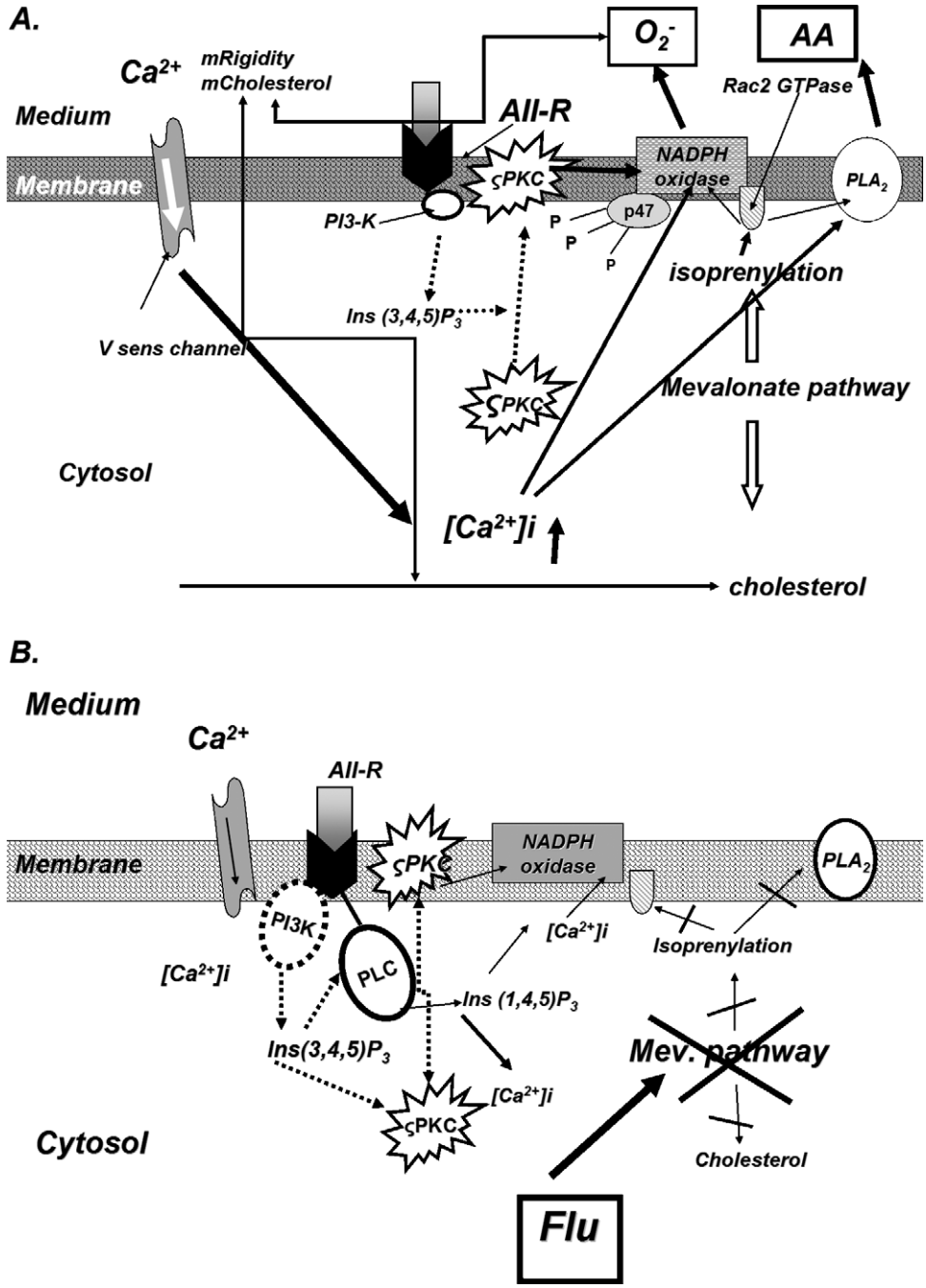

Fig. 4. One of the possible signaling pathways through AT1 receptors in neutrophils of patients with HC (A) and Flu-treated patients (B). AA indicates arachidonic acid cascade; AII-R, angiotensin II receptor; $IP_3(1,4,5)$, $Ins(1,4,5)P_3$; $IP_3(3,4,5)$, $Ins(3,4,5)P_3$; m, membrane bound; Mev, mevalonate.

synthesis. Furthermore, Ca^{2+} signal and diacylglycerol cause PKC activation and translocation into the membrane [39]. According to our recent and earlier findings, in neutrophils of patients with HC, the increase in free radical generation is caused by changes of intracellular signal transduction [1,14,39]. One of the possible alternative signaling pathways is demonstrated in Fig. 4A. In neutrophils of patients with HC, the failure of PT-sensitive IP_3 and IP_3-induced Ca^{2+} signals suggest that the AT1 receptor—Gi protein—PLC pathway is injured, and the signaling occurs in part through the opening of the V-sensitive Ca^{2+} channels. As a consequence of altered membrane rigidity and composition in neutrophils of patients with HC, one of the possibilities is a second alternative signaling pathway through the activation of PI3K-Ins(3,4,5)P_3, the latter being able to trigger translocation of PKCζ into the membrane [40]. On the other hand, in some patients with HC, the increase in the LDL cholesterol level in the serum is caused by the enhancement of endogenous cholesterol synthesis through increased activity of the mevalonate pathway. However, the elevated activity of mevalonate pathway results in an increase in the isoprenylation of Rac2 GTPase and, consequently, NADPH oxidase and PLA_2 activities [6]. In Fig. 4B, the partial restoration of signaling achieved by Flu administration is demonstrated. The inhibition of Rac2 GTPase isoprenylation dramatically decreases NADPH oxidase and PLA_2 activities. In the Flu-treated group, the appearance of either the PT-resistant IP_3 signal or both the V-sensitive and V-insensitive Ca^{2+} signals suggests that the genuine signaling pathway in neutrophils is not restored by Flu therapy. In addition, the partial restoration of membrane-bound PKC activity and the unchanged membrane rigidity can serve as evidence for the existence of alternative signaling [41].An explanation for the partially restored signaling can be that the PI3K-produced Ins(3,4,5)P_3 is able to trigger PLC and, consequently, the PT-resistant IP_3 and Ca^{2+} signals [42]. It should be pointed out that we could not exclude other alternative pathways such as signaling through MAP kinase [43,44] or through PT-resistant Gq protein [45]. Finally, it should be noted that membrane-bound cholesterol content and, consequently, membrane fluidity can alter cholecystokinin-β and oxytocin receptor function and that we may not rule out a direct interaction between membrane cholesterol and AII receptors either [46].

Before summarizing the results of our clinical trials, it must be emphasized that these experiments were conducted in a parallel manner and were not set up as self-controlled systems. Consequently, some caution is advisable at their evaluation. We conclude that Flu administration counteracts the AII-triggered enhanced free radical production of neutrophils obtained from patients with HC through the inhibition of Rac2 GTPase. Furthermore, Flu administration has an effect on the signal processing of AII as well as on the enzymes and ion channels, which are in close relationship with the membrane. In spite of the lowering-cholesterol level in plasma and cell membrane, the increased membrane rigidity remains unchanged after Flu administration, possibly as a consequence of the Flu-resistant phospholipid composition of the cell membrane caused by HC. Therefore, it cannot rule out the possibility that there is a connection between cell membrane rigidity and the altered functions of membrane-bound components of the neutrophils of patients with HC. These components are involved in altered signal processing after AII stimulation, and this altered signal processing cannot be completely restored with Flu administration in patients with HC.

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