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In human hypercholesterolemia increased reactivity of vascular smooth muscle cells is due to altered subcellular Ca^{2+} distribution

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Abstract

There is evidence that, besides an attenuated endothelium-dependent relaxation, functional changes in smooth muscle contractility occur in experimental hypercholesterolemic animals. Unfortunately, little is known of the situation in human arteries, and the intracellular mechanisms involved in the modulation of vascular smooth muscle function in human hypercholesterolemia are still unclear. Thus, besides acetylcholine-induced endothelium-dependent relaxation, smooth muscle reactivity to KCl, norepinephrine (NE) and phenylephrine (PE) was evaluated in uterine arteries from 34 control individuals (CI) and 22 hypercholesterolemic patients (HC). Contractions to KCl, norepinephrine and phenylephrine were enhanced by 1.3-, 2.1- and 3.5-fold in vessels from HC. Furthermore, the Ca^{2+} signaling in the perinuclear cytosol, which promotes cell contraction, and that of the subplasmalemmal region, which contributes to smooth muscle relaxation, were examined in freshly isolated smooth muscle cells. In cells from HC, increases in perinuclear Ca^{2+} concentration ($[Ca^{2+}]_{peri}$) in response to 30 mM KCl and 300 nM NE were increased by 67 and 93%, respectively. In contrast, the increase in the subplasmalemmal Ca^{2+} concentration ($[Ca^{2+}]_{sub}$) to 10 μM NE was reduced in cells from HC by 33%. No further differences in perinuclear and subplasmalemmal Ca^{2+} signaling were found in cultured smooth muscle cells from CI and HC (primary culture 4–6 weeks after isolation). These data indicate a significant change in the subcellular Ca^{2+} distribution in smooth muscle cells from HC. In addition, production of superoxide anions (O_2^-) was increased 3.8-fold in uterine arteries from HC. Treatment of smooth muscle cells with the O_2^--generating mixture xanthine oxidase/hypoxanthine mimicked hypercholesterolemia on smooth muscle Ca^{2+} signaling. From these findings, we conclude that during hypercholesterolemia, besides a reduced endothelium-dependent relaxation, changes in smooth muscle reactivity take place. Thereby, smooth muscle contractility is increased possibly due to the observed changes in subcellular Ca^{2+} signaling. The observed increased O_2^- production in HC might play a crucial role in the alteration of smooth muscle function in hypercholesterolemia.

Keywords: Ca^{2+} distribution; Contractility; Norepinephrine; Phenylephrine; Superoxide anions

1. Introduction

Hypercholesterolemia is associated with an increased risk for vascular complications and atherosclerosis [1]. In contrast to consistent data indicating a reduced endothelium-dependent relaxation in hypercholesterolemic humans [2] and animals [3], the reports on the effect of hypercholesterolemia on vascular smooth muscle responsiveness are controversial. While the angiotensin II- and methoxamine-induced contraction of the aorta was reduced in hypercholesterolemic rabbits [4,5], experimental hypercholesterolemia enhanced vasoconstriction to 5-hydroxytryptamine in porcine coronary arteries [6] and that of cavernosal smooth muscles to norepinephrine (NE) [7]. Moreover, in hypercholesterolemic animals smooth muscle responsiveness to the relaxing nitric oxide reduces, while contractility to NE and endothelin was increased [8,9].

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It has been suggested recently, that the increased smooth muscle reactivity in hypercholesterolemia is due to an enhanced intracellular Ca^{2+} signaling to contractile stimuli [9]. In smooth muscle cells the Ca^{2+} concentrations of at least two compartments of the cytoplasm differentially affect the tone [10–13]. An increase in the perinuclear Ca^{2+} concentration ($[Ca^{2+}]_{peri}$) promotes contraction [14], while elevated subplasmalemmal Ca^{2+} concentrations ($[Ca^{2+}]_{sub}$) favors smooth muscle relaxation due to activation of Ca^{2+}-activated K^+ channels [15]. In regard of the divergent contributions of these localized Ca^{2+} signals for smooth muscle reactivity it needs to be investigated in more detail which aspect of the Ca^{2+} signaling (i.e. $[Ca^{2+}]_{peri}$ or $[Ca^{2+}]_{sub}$) is affected by hypercholesterolemia. In hypercholesterolemic rabbits the enhanced sensitivity of the vascular smooth muscle cells to contract to KCl and NE was normalized by the free radical scavenger probucol [16]. These findings are in line with other reports indicating that superoxide dismutase (SOD) or antioxidative vitamins, such as vitamins E, normalized vascular reactivity in hypercholesterolemic animals [17]. In agreement with these reports, an increased formation/release of O_2^- in hypercholesterolemic rabbits has been described [18,19]. Thus, in hypercholesterolemic animals there is evidence that the changes in smooth muscle cell reactivity are associated with an increased appearance of reactive oxygen species. However, limited information has been provided on the situation in humans and how these pathological events may be or may not be interrelated.

This study was designed to investigate, in freshly isolated human arteries, whether hypercholesterolemia is associated with a hyperreactivity of arteries to KCl and NE/phenylephrine (PE) and an increased O_2^- release. The effect of hypercholesterolemia on the smooth muscle Ca^{2+} signaling in the perinuclear and the subplasmalemmal areas of the cytoplasm was assessed. Thus, the vascular smooth muscle was explored as a target in hypercholesterolemia, which may contribute to the development of vascular complications and atherosclerosis in hypercholesterolemic men.

Table 1
Clinical characteristics of the control and hypercholesterolemic groups[a]

	Units	CI ($n=42$)	HC ($n=27$)
Age	years	57.3 ± 13.8	55.7 ± 7.9
Body mass	kg/m^2	21.4 ± 2.3	22.6 ± 3.1
Blood pressure (systolic)	mmHg	121 ± 10	137 ± 24
Blood pressure (diastolic)	mmHg	80 ± 12	87 ± 8
HbA_{1c}	%	5.0 ± 1.2	5.6 ± 0.5
D-Glucose	mg/dl	93.4 ± 10.1	97.4 ± 13.2
Cholesterol	mg/dl	193.2 ± 28.1	263.2 ± 27.2*
LDL-Cholesterol	mg/dl	112.7 ± 25.2	169.4 ± 19.1*
HDL-Cholesterol	mg/dl	58.0 ± 19.1	60.7 ± 13.1
Triglycerides	mg/dl	105.4 ± 39.6	143.4 ± 75.9
Creatinine	mg/dl	0.84 ± 0.15	0.84 ± 0.11
GOT	U/l	8.3 ± 1.2	12.1 ± 5.1

[a] Mean ± S.D.
* $P<0.05$ HC vs CI (evaluated by ANOVA following a post hoc analysis with Scheffe's F-test).

2. Methods

2.1. Subjects

Uterine arteries from postmenopausal women undergoing hysterectomy for treatment of uncomplicated, uterus myomatosus were used. Permission of the donors of the arteries was obtained after the donors had been informed extensively on the proposed work. As shown in Table 1, the characteristics of control individuals (CI) and hypercholesterolemic patients (HC) differed only in terms of total cholesterol and LDL-cholesterol (HC). None of the subjects had drug therapy.

2.2. Vessel preparation

The ascending main branch of the uterine artery was dissected from connective tissue and cut in ring segments of 3 mm diameter and 2 mm in length for organ chamber experiments. Wall thickness in each group did not differ and only vessels with no visible plaques were used. Air was blown through the intact vessels, followed by a metal probe into the lumen to remove the endothelium. Efficiency of endothelium removal was tested by the lack of relaxation to 1 μM A23187.

2.3. Cell culture

Smooth muscle cells were isolated by cutting a 3-cm piece of the artery longitudinally. The vessel was fixed in an incubation frame. The endothelium was removed and the vessel was incubated under sterile conditions for 16 h at room temperature in Dulbeccos's minimal essential medium (DMEM; pH 7.4) containing collagenase (200 U/ml; type II), plus dilutions of essential and non-essential amino acids (0.02 v/v; Gibco-BRL, Life Technologies, Vienna, Austria), vitamins (0.01 v/v; Gibco-BRL), donor horse serum (5%) and bovine serum albumin (2 mg/ml). The suspended cells were used for the experiments or were cultured up to passage 2 in Opti-MEM containing 5% fetal calf serum and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin and 1.25 μg/ml fungizone) at 37°C under 5% CO_2 atmosphere and 95% humidity. Cell culture purity was tested by typical valley/hill morphology of the confluent dish and by staining smooth muscle α-actin.

2.4. Organ chamber experiments

Blood vessel contractility was tested according to our previous technique [20]. Briefly, ring segments were mounted in an organ bath with 2 ml of a Krebs–Henseleit solution (KHS; in mM: 118.4 NaCl, 5.01 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25.0 $NaHCO_3$; pH adjusted at 7.4) constantly gassed with 95% O_2 and 5% CO_2 at 37°C. Isometric tension was monitored using a force-displacement transducer coupled to a bridge amplifier and tension was continuously recorded with a foue-channel printer. Rings were allowed to equilibrate for 90 min in the bath, while the solution was replaced every 15 min. Prior to the experiments, vessels were precontracted three times to 2 g. Cumulative concentration response curves for the compounds to be tested were performed after the vessel reached a stable tone.

2.5. Ca^{2+} measurements

Free intracellular Ca^{2+} concentration was examined in freshly isolated and cultured single human smooth muscle cells using a microfluorometric set-up described previously [21]. The *perinuclear free Ca^{2+} concentration* ($[Ca^{2+}]_{peri}$) was measured with fura-2 and the *subplasmalemmal Ca^{2+} concentration* ($[Ca^{2+}]_{sub}$) was monitored with FFP-18 as described previously [22,23]. Smooth muscle cells were loaded by incubating the cells in DMEM containing 2 μM fura-2/am for 45 min at room temperature. FFP-18 was loaded using the laser stress wave loading technique, described recently [22]. Briefly, approximately 10^6 cells were suspended in phenol red-free DMEM containing 2 μM FFP-18 and transferred into a microtiterplate. After the well had been sealed with a 1.25-mm aluminum foil, laser-induced stress waves were generated by three to five shots of a Nd:YAG laser (70 mJ) to the metal foil, focused 0.5 cm behind the foil. After an equilibration time of 20 min in the dark at room temperature, cells were centrifuged and resuspended in DMEM. For fura-2 experiments, cells were transferred into an experimental chamber and superfused with KHS or nominal Ca^{2+}-free KHS containing 10 μM EGTA by constant superfusion (1 ml/min). For FFP-18 experiments, cells were resuspended in KHS and transferred into a stirred cuvette.

2.5.1. Data acquisition

2.5.1.1. Fura-2. In single cells $[Ca^{2+}]_{peri}$ was monitored using a microfluorometer [24] and fura-2 was alternatively excited with 360 and 380 nm while emission light was detected at 510 nm [21]. Fluorescence intensity for each pair of excitation/emission wavelengths was converted to analog by an optical processor [24] and registered by a PC running AxoBASIC® 1.0 (Axon Instruments, Foster City, CA, USA).

2.5.1.2. FFP-18. Changes in $[Ca^{2+}]_{sub}$ were monitored in suspended cells every 0.25 s at 335 and 364 nm excitation, at 490 nm emission, in a stirred cuvette using a four-wavelength spectrofluorometer (Hitachi F4500).

2.5.2. Data analysis

Due to the overall failure and the reported uncertainties in the calibration for intracellular Ca^{2+} concentration [21,24,25], Ca^{2+} concentration in each experiment is expressed as ratio 360/380 excitation at 510 nm emission (F_{360}/F_{380}; fura-2) or the ratio 335/364 nm excitation and 490 nm emission (F_{335}/F_{364}; FFP-18). Maximal fluorescence intensities of either fura-2 or FFP-18 to verify possible differences in dye distribution/accumulation were measured after each experiment by adding 3 μM ionomycin in the presence of 2.5 mM Ca^{2+}. Minimal fluorescence and autofluorescence were monitored in Ca^{2+}-free solution containing 3 μM ionomycin and either 1 mM EGTA or Mn^{2+}. To allow comparison of the effect with other reports, intracellular Ca^{2+} signaling was calibrated using the in situ calibration technique described by Sturek et al. [24]

2.6. Measurement of superoxide anions

The release of superoxide anions (O_2^-) was determined by measuring the superoxide dismutase (SOD)-sensitive reduction of ferrocytochrome c [26]. The wet weight of ring segments of the arteries with or without endothelium was determined (approximately 20 mg each). Rings were incubated in phosphate-buffered solution (in mM: 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4 and 0.1 EGTA; pH adjusted at 7.4) containing 10 μM ferrocytochrome c and the reduction of ferrocytochrome c was followed at 550 nm over 30 min. To ensure that the observed reduction of the ferrocytochrome c was due to O_2^-, 500 U/ml SOD (from bovine erythrocytes) were added and the reduction of ferrocytochrome c was monitored for a further 30 min. The difference in absorption between the samples without and with SOD was calculated, and indicated the SOD-sensitive reduction of ferrocytochrome c. The concentration of O_2^- was calculated using the molar extinction coefficient ($\varepsilon = 21.000$; [27]) of the reduced ferrocytochrome c.

2.7. Materials and drugs

Petri dishes and cell culture plastic wear were obtained from Corning (Vienna, Austria). Cell culture chemicals were purchased from Life Technologies (Vienna), and fetal calf serum (premium quality) and

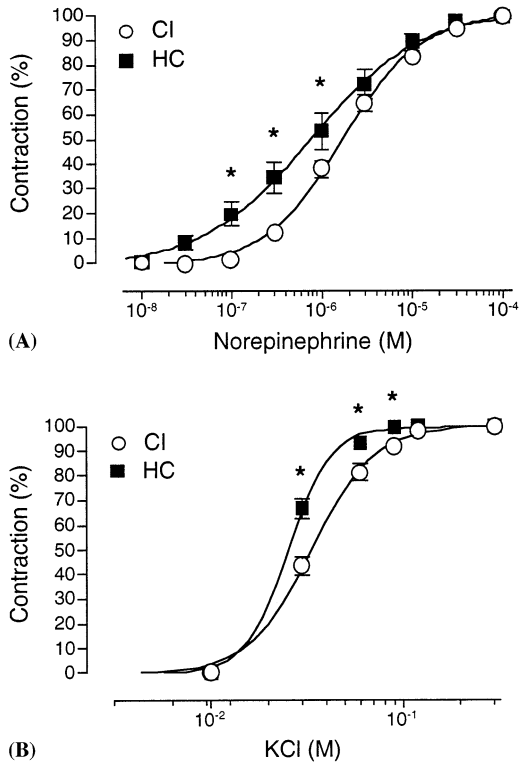

Fig. 1. Concentration contraction relationship to NE (A) and increased extracellular K^+ concentration (B) in uterine arteries isolated from CI (open symbols; $n = 13$) and HC (filled squares; $n = 8$). Changes in smooth muscle tone were isometrically recorded performing a cumulative concentration response curve for NE. Values represent the mean ± S.E.M. of contraction achieved with the indicated concentration of NE calculated in percent of the maximal contraction to NE (100 μM; A) and in percent of the maximal contraction to K^+ (B). *$P < 0.05$ versus contraction in CI.

donor horse serum were from PAA Laboratories (Linz, Austria). Fura-2/am was purchased from Molecular Probes (Leiden, The Netherlands) and FFP-18 was from Calbiochem (Vienna). Buffer salts were from Merck (Vienna), and all other materials were purchased from Sigma (Vienna).

2.8. Statistical analysis

The given '*n*' values express the number of patients whose uterine arteries and freshly isolated smooth muscle cells were tested in triplicate to quadruplicate (contraction studies) and quintuplicate to nonaduplicate (Ca^{2+} experiments). The EC_{50} values are expressed as the mean with 95% confidential intervals in parenthesis. Data represent the mean ± S.E.M. Analysis of variance (ANOVA) was used for data evaluation including post hoc verification using the Turnkey test. Differences were considered to be statistical significant at $P < 0.05$.

3. Results

3.1. Contractility of normal and hypercholesterolemic vessels

In freshly isolated human uterine arteries from CI and HC a concentration response curve for norepinephrine (NE)-induced contraction was performed. The EC_{50} of NE for vasoconstriction was 1.69 (1.51–1.88) μM in the control group and 0.79 (0.58–1.09) μM in the hypercholesterolemic group (Fig. 1A; $P < 0.05$ for HC vs Cl). In addition, the EC_{50} values for phenylephrine (PE)-induced contraction were reduced in vessels from HC from 10.69 (9.10–12.55) to 3.02 (2.46–3.71) μM ($P < 0.05$ for HC vs CI).

Besides the receptor-dependent agonists, NE and PE, contractility of uterine arteries to non-receptor-dependent mechanism were assessed by studying blood vessel contraction to KCl (Fig. 1B). Similar to our data on NE and PE, the EC_{50} values of the vessels to contract to KCl was reduced from 33.63 (32.30–35.01) mM extracellular K^+ in the CI to 25.29 (24.41–26.20) mM in the HC (Fig. 1B; $P < 0.05$ vs for HC vs CI).

While the sensitivity of arteries isolated from HC to contract to NE, PE and increased extracellular K^+ concentration was increased compared with that obtained in vessels from CI, maximal contraction achieved by each stimulus did not differ in either groups (KCl: CI, 3.11 ± 0.43 g, HC, 3.22 ± 0.56 g; NE: CI, 4.56 ± 0.39 g, HC, 4.21 ± 0.44 g and PE: CI, 5.44 ± 0.72 g, HC, 4.02 ± 0.91 g).

3.2. Dilatory properties of normal and hypercholesterolemic vessels

Endothelium-dependent relaxation in response to acetylcholine was tested in arteries from CI and HC (Fig. 2). Acetylcholine-induced relaxation was significantly reduced in arteries from HC (CI, 56.3 ± 0.6% vs HC, 33.6 ± 0.2%; $P < 0.005$; Fig. 2). The EC_{50} value for acetylcholine to initiate endothelium-dependent relaxation was slightly increased in the HC group (CI, 57.2 (37.8–86.6) nM); HC, 264.3 (208.8–334.7) nM). In endothelium-denuded vessels no relaxation in response to 1 μM A23187 or 1 μM acetylcholine was found (data not shown).

3.3. Perinuclear Ca^{2+} signaling in single smooth muscle cells from CI and HC

Freshly isolated smooth muscle cells were stimulated in the presence of 2.5 mM extracellular Ca^{2+} with either KCl (30 and 60 mM; Fig. 3A, upper panel) or 0.3 and 10 μM NE (Fig. 3B, upper panel). Moreover, cells were activated with 10 μM NE in the nominal absence of extracellular Ca^{2+} followed by the addition of 2.5

mM Ca^{2+} (Fig. 3C, upper panel). Changes in $[Ca^{2+}]_{peri}$ were monitored in single smooth muscle cells. Compared with the corresponding elevation in $[Ca^{2+}]_{peri}$ in cells from CI, the increase in $[Ca^{2+}]_{peri}$ in response to 30 and 60 mM KCl was augmented in cells from HC by 73 and 52%, respectively ($P < 0.05$ versus the effect obtained in CI; Fig. 3A, lower panel). In agreement with the enhanced elevation in $[Ca^{2+}]_{peri}$ to KCl in smooth muscle cells from HC, the effect of 0.3 and 10 μM NE was greater in HC compared with CI (Fig. 3B, lower panel). Hence, intracellular Ca^{2+} release evoked by 10 μM NE in nominal Ca^{2+}-free KHS was increased by 87% in cells from HC (Fig. 3C, lower panel). The addition of 2.5 mM Ca^{2+} to pre-stimulated cells from HC-induced greater increase in $[Ca^{2+}]_{peri}$ than in cells from CI (Fig. 3C, lower panel). Similar to our results with NE, PE-evoked elevation in $[Ca^{2+}]_{peri}$ in the presence and absence of extracellular Ca^{2+} was increased in smooth muscle cells freshly isolated from HC compared with that from CI (data not shown).

In contrast to the effect of NE/PE/KCl on smooth muscle Ca^{2+} signaling, elevation in $[Ca^{2+}]_{peri}$ to 3 μM ionomycin in the presence of 2.5 mM extracellular Ca^{2+} did not differ between cells isolated from CI and HC (data not shown). In addition, minimal fluorescence and autofluorescence in cells isolated from CI did not differ from that derived from HC patients. These findings indicate that the reported differences in agonist-induced Ca^{2+} signaling are not due to alterations in dye loading, distribution or sensitivity.

3.4. Perinuclear Ca^{2+} signaling in cultured single smooth muscle cells from CI and HC

In contrast to our findings in the freshly isolated smooth muscle cells, there was no difference between the KCl- and NE-evoked perinuclear Ca^{2+} signaling in cultured single smooth muscle cells from CI and HC (primary culture 4–6 weeks after isolation). Identical experimental protocols as shown in the upper panels of Fig. 3 were used. In the protocol shown in upper panel of Fig. 3A, the increase of $[Ca^{2+}]_{peri}$ in cultured cells from CI in response to 30 and 60 mM KCl (0.015 ± 0.001 and 0.027 ± 0.001 ratio (F_{360}/F_{380}) units, $n = 23$) was very similar to that obtained in cultured cells from HC (0.016 ± 0.001 and 0.031 ± 0.002 ratio (F_{360}/F_{380}) units; $n = 25$, ns vs cells from CI). In addition, no differences in the response to 0.3 and 10 μM NE in the presence of external Ca^{2+} (according to the protocol shown in upper panel of Fig. 3B; $n = 57–63$) and NE-induced intracellular Ca^{2+} release followed by the addition of 2.5 mM Ca^{2+} (according to the protocol shown in upper panel of Fig. 3C) were found in cultured cells from CI and HC ($n = 36–42$; data not shown). In agreement with these findings, no difference was observed in the effect of 10 μM PE on $[Ca^{2+}]_{peri}$ in cultured cells isolated from CI and HC (data not shown).

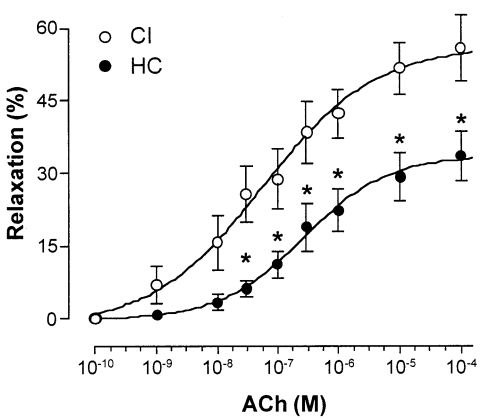

Fig. 2. Cumulative concentration–response curves of the relaxing effects of acetylcholine in uterine arteries from CI (open circles; $n = 7–9$) and HC (filled circles; $n = 7–9$). Intact rings were precontracted with 30 mmol/l KCl. Changes in tension to the acetylcholine concentration indicated were measured isometrically and calculated as percentage of the reduction of the KCl-induced tone. Values represent the mean ± S.E.M. of relaxation achieved with the acetylcholine concentration indicated. $^*P < 0.05$ versus relaxation in CI.

To test whether the composition of the sera might contribute to the observed changes in smooth muscle Ca^{2+} signaling, cultured human smooth muscle cells isolated from CI were incubated for 24 h in DMEM containing 10% serum isolated from either CI or HC, and the effects of 10 μM PE and 30 mM KCl on $[Ca^{2+}]_{peri}$ were assessed. In cells incubated with serum from HC perinuclear Ca^{2+} signaling to 10 μM PE was augmented by 51% ($P < 0.05$; Fig. 4). Co-incubation with 300 U/ml SOD reduced the effect of serum from HC patients on PE-induced Ca^{2+} signaling ($P < 0.05$; Fig. 4). Similar to the increased response to PE in cells treated with serum from HC patients, elevation in $[Ca^{2+}]_{peri}$ in response to 30 mM extracellular KCl was increased in cells treated with HC serum (data not shown).

Removal of lipoproteins by gradient centrifugation (bottom-fraction after gradient ultracentrifugation of donor serum set at 1.21 density with NaBr with 48 000 rpm for 48 h at 15°C) prevented the effect of serum from HC patients on smooth muscle Ca^{2+} signaling in response to 10 nM endothelin-1 (data not shown).

3.5. Subplasmalemmal Ca^{2+} signaling in smooth muscle cells freshly isolated from CI and HC

In contrast to the increased perinuclear Ca^{2+} signal found in cells from HC (Fig. 3), the effect of 10 μM NE on $[Ca^{2+}]_{sub}$ was reduced by 33% in smooth muscle cells freshly isolated from HC compared with that from CI (Fig. 5A). Despite these observed differences in subplasmalemmal Ca^{2+} signaling, the effect of 3 μM

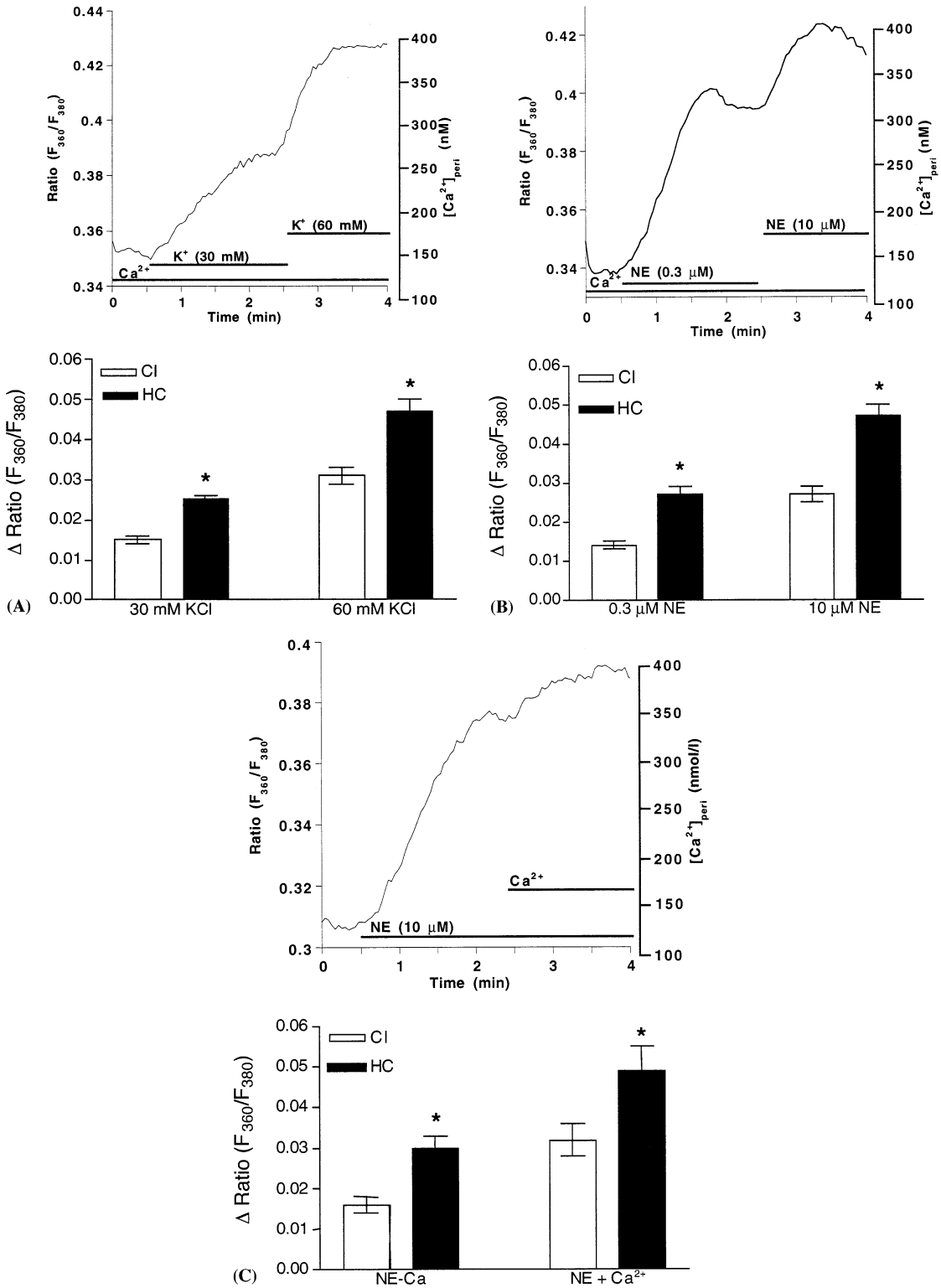

Fig. 3. Measurements of perinuclear Ca^{2+} response in single smooth muscle cells freshly isolated from uterine arteries of CI (open bars; $n = 13$) and HC (filled bars; $n = 8$). Cells were loaded with fura-2/am to monitor changes in $[Ca^{2+}]_{peri}$ in KHS containing 2.5 mM Ca^{2+} (A, B) and in nominal Ca^{2+}-free KHS (C). Experimental protocols are shown in the upper graph of each panel and the results are given in the lower graph as the observed maximal changes of each stimulus (Δratio (F_{360}/F_{380})). *$P < 0.05$ versus the effect obtained in the CI group using the same experimental protocol.

ionomycin in the presence of 2.5 mM extracellular Ca^{2+} was identical in both groups, indicating that the observed differences are unlikely to be due to alterations in dye loading, sensitivity or distribution. In cultured smooth muscle cells (primary culture 4–6 weeks after isolation), isolated from DM and HC, no difference in NE (10 μM) evoked elevation of $[Ca^{2+}]_{sub}$ was observed (data not shown).

In agreement with our findings in freshly isolated cells, a 24-h incubation of cultured smooth muscle cells with 10% serum from HC decreased subplasmalemmal Ca^{2+} elevation in response to 10 μM NE by 21% (Fig. 5B).

3.6. O_2^- generation in normal and hypercholesterolemic vessels, with and without endothelium

Release of O_2^- measured as the SOD-sensitive reduction of ferrocytochrome *c* was determined in uterine arteries derived from CI and HC. Compared with the release of O_2^- measured in CI, in HC the release of O_2^- was enhanced by 286% (Fig. 6A). Removal of the endothelium slightly reduced O_2^- release in hypercholesterolemic vessels, while the release of O_2^- remained to be enhanced in the hypercholesterolemic vessels by 216% (Fig. 6B).

3.7. Effect of an O_2^- treatment on smooth muscle Ca^{2+} signaling

To test whether the enhanced O_2^- production might contribute to the altered Ca^{2+} signaling in freshly isolated smooth muscle cells from HC, freshly isolated smooth muscle cells from CI were incubated with a mixture of 400 μU xanthine oxidase and 1 mM hypoxanthine for 4 h. This mixture produces approximately the same amount of O_2^- (i.e. 6.53 ± 0.82 μmol/10 min) that was found in the hypercholesterolemic vessels (Fig. 5A). Preincubation of smooth muscle cells with the O_2^--generating mixture increased the effect of 10 μM NE on $[Ca^{2+}]_{peri}$ by 72% (Fig. 7A). In contrast, treatment with the O_2^--generating system reduced NE-initiated elevation in $[Ca^{2+}]_{sub}$ by 24% (Fig. 7B).

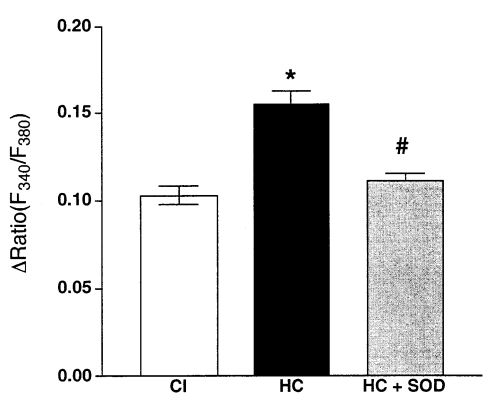

Fig. 4. Perinuclear Ca^{2+} signaling to PE in cultured smooth muscle cells incubated for 24 h in DMEM containing 10% serum isolated from CI (open bar, $n = 7$) and HC (filled bar, $n = 7$) in the absence (CI, HC) and presence of 300 U/ml SOD (HC + SOD; $n = 6$). Cells were loaded with fura-2. In KHS containing 2.5 mM Ca^{2+} changes cells were stimulated with 10 μM PE. Maximal changes in $[Ca^{2+}]_{peri}$ are given as the Δ values of the ratio (F_{340}/F_{380}). $^*P < 0.05$ versus the effect obtained in cells incubated with serum from CI. $^{\#}P < 0.05$ versus the effect obtained in cells incubated with serum from HC.

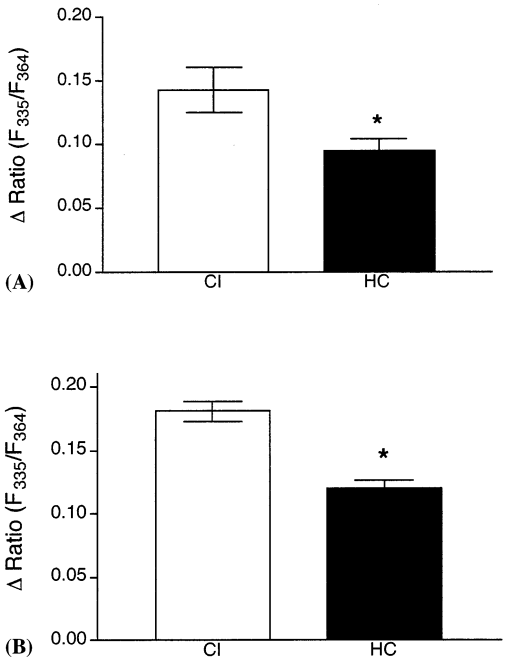

Fig. 5. Subplasmalemmal Ca^{2+} signaling to NE in smooth muscle cells freshly isolated from uterine arteries obtained from CI and HC (A) and cultured cells incubated with serum from CI or HC (B). Cells were loaded with FFP-18 using the laser pulse wave-loading technique described in Section 2. (A) Freshly isolated smooth muscle cells were obtained from CI (open bar; $n = 13$) and HC (filled bar; $n = 8$). In KHS containing 2.5 mM Ca^{2+} cells were stimulated with 10 μM NE and $[Ca^{2+}]_{sub}$ was monitored at 335 and 364 nm excitation and 490 nm emission. Maximal changes in $[Ca^{2+}]_{sub}$ are given as the Δ values of the ratio (F_{335}/F_{364}). $^*P < 0.05$ versus the effect obtained in cells isolated from CI. (B) Cultured smooth muscle cells from CI were incubated for 24 h in DMEM containing 10% serum isolated from CI (open bar, $n = 4$) and HC (filled bar, $n = 6$). In KHS containing 2.5 mM Ca^{2+} changes cells were stimulated with 10 μM PE. Maximal changes in $[Ca^{2+}]_{peri}$ are given as the Δ values of the ratio (F_{340}/F_{380}). $^*P < 0.05$ versus the effect obtained in cells incubated with serum from CI.

4. Discussion

The present work demonstrates an increased sensitivity to contractile stimuli and a reduced endothelium-dependent relaxation in vessels from humans with hypercholesterolemia. These findings are in agreement with reports collected in experimental hypercholesterolemia in animals [8,9,30,31]. Since hypercholes-

terolemia enhanced vasoconstriction in response to receptor-dependent (i.e. the α_1-agonists NE and PE) and

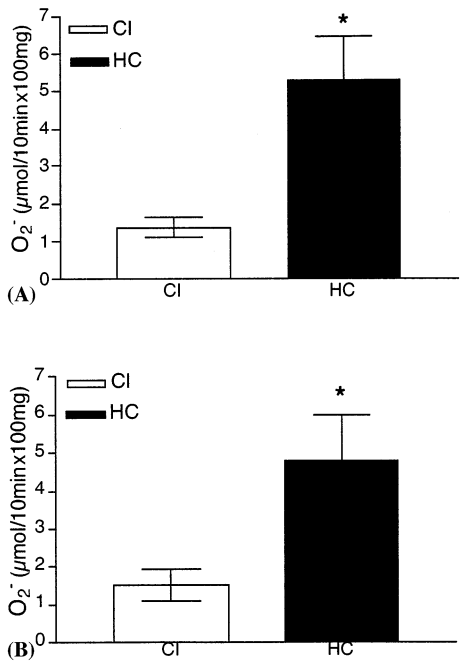

Fig. 6. Release of O_2^- from fresh uterine arteries obtained from CI (open bar; $n = 13$) and HC (filled bar; $n = 8$) with (A) and without (B) endothelium. O_2^- release was monitored photometrically by the reduction of ferrocytochrome c as described in Section 2. $^*P < 0.05$ versus O_2^- production measured in arteries of CI.

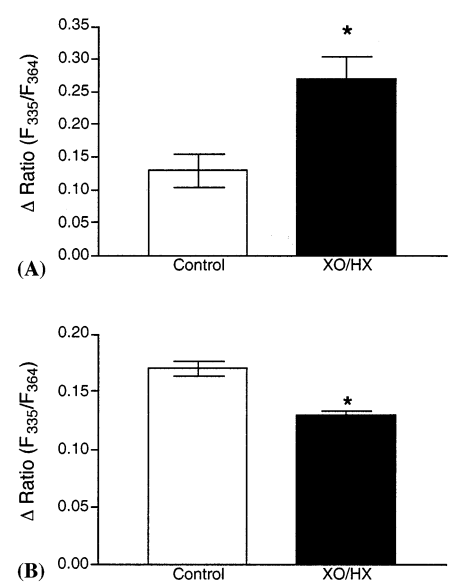

Fig. 7. Effect of superoxide anion treatment on perinuclear (A) and subplasmalemmal (B) Ca^{2+} signaling in freshly isolated smooth muscle cells from CI. Freshly isolated cells were incubated for 4 h in DMEM containing 400 µU xanthine oxidase/1 mM hypoxanthine (XO/HX). Cells were loaded with fura-2 (A) or FFP-18 (B) as described in Section 2, and maximal changes in $[Ca^{2+}]_{peri}$ (A) and $[Ca^{2+}]_{sub}$ (B) in response to 10 µM NE in the presence of 2.5 mM extracellular Ca^{2+} were monitored. $^*P > 0.05$ versus control: (A) control, $n = 5$, XO/HX, $n = 6$; (B) control, $n = 6$, XO/HX, $n = 9$).

receptor-independent (i.e. KCl) stimuli, one might speculate that hypercholesterolemia affects a common intracellular mediator (i.e. Ca^{2+}) for α-agonist- and KCl-evoked contraction. In vascular smooth muscle cells, localized elevation of $[Ca^{2+}]_{sub}$ has been demonstrated which is insulated from $[Ca^{2+}]_{peri}$ and contributes to smooth muscle relaxation, while increases in $[Ca^{2+}]_{peri}$ favor smooth muscle contraction [10–13]. In contrast to fura-2 that monitors $[Ca^{2+}]_{peri}$ [22,23], the fura-2 derivate FFP-18 is primarily localized in the cell membrane [23,28,29] and, thus, it allows to measure exclusively $[Ca^{2+}]_{sub}$ [22,23]. Therefore, our findings that in single smooth muscle cells from HC an augmented increase in $[Ca^{2+}]_{peri}$ in response to increased KCl, NE and PE occurred, suggest that an increased elevation in $[Ca^{2+}]_{peri}$ to KCl, NE and PE contributes to the observed enhanced contractility to these autacoids. These data are in line with reports in hypercholesterolemic rabbits [9,16], where an augmented perinuclear Ca^{2+} signaling to KCl and NE has been demonstrated. Additional information on the effect of hypercholesterolemia on smooth muscle Ca^{2+} signaling provided by our experiments demonstrating an enhanced elevation in $[Ca^{2+}]_{peri}$ in response to NE in the absence of extracellular Ca^{2+} (Fig. 2C), pointing to an enhanced autacoid-evoked (inositol 1,4,5-trisphosphate-mediated) intracellular Ca^{2+} release in hypercholesterolemic smooth muscle cells. Moreover, the agonist-stimulated Ca^{2+} entry indicated by the effect of an addition of extracellular Ca^{2+} to NE stimulated cells was also increased in smooth muscle cells from HC. Thus, perinuclear Ca^{2+} elevations in response to either intracellular Ca^{2+} release (NE,PE) or Ca^{2+} entry (KCl, NE, PE) were found to be increased in cells from HC, suggesting that the reported changes in the perinuclear Ca^{2+} signaling does not depend on the mechanism responsible for the increase in $[Ca^{2+}]_{peri}$. In contrast to the observed enhancement in the perinuclear Ca^{2+} signaling to KCl and NE in smooth muscle freshly isolated from HC, intracellular Ca^{2+} signaling to KCl and NE was similar in cultured smooth muscle cells from HC compared with that obtained in cultured smooth muscle cells from CI. Remarkably, an incubation of cultured smooth muscle cells with serum from HC mimicked the situation in freshly isolated smooth muscle cells from these patients and increased intracellular Ca^{2+} signaling as compared with cells incubated with serum from CI. These data and our findings that removal of lipoproteins from the serum abolished the influence of hypercholesterolemic serum on smooth muscle Ca^{2+} signaling are in line with dogma that, in hypercholesterolemia, the changes in serum composition (high LDL levels, oxidized LDL, lysophosphatidylcholine) initiate vascular cell dysfunction and vascular complication [32].

In contrast to our findings on the enhanced perinuclear Ca^{2+} signaling in hypercholesterolemic smooth muscle cells, subplasmalemmal Ca^{2+} response was reduced in HC. Thus, our data indicate changes in the Ca^{2+} signaling in the two major regulatory sites for smooth muscle tone during hypercholesterolemia. Remarkably, while autacoid-induced elevation of the $[Ca^{2+}]_{peri}$ which triggers smooth muscle contraction to K^+ and NE, was increased in hypercholesterolemic smooth muscle cells, subplasmalemmal Ca^{2+} response was reduced. Such divergent changes in the subplasmalemmal and perinuclear Ca^{2+} signaling in HC might be of particular importance in respect of the differential role of $[Ca^{2+}]_{sub}$ and $[Ca^{2+}]_{peri}$ for smooth muscle tone. While $[Ca^{2+}]_{sub}$ has been reported to promote relaxation by activation of Ca^{2+}-activated K^+ channels and to constitute a negative feedback for smooth muscle contraction [15], an increase in $[Ca^{2+}]_{peri}$ favors smooth muscle contraction [14]. On the basis of our data, one might speculate that in hypercholesterolemia the attenuated elevation of $[Ca^{2+}]_{sub}$ upon cell activation results in a reduced activation of Ca^{2+}-activated K^+ channels and, thus, in an attenuated inhibitory control for contraction. Therefore, our data on human hypercholesterolemia-associated alterations in smooth muscle Ca^{2+} signaling suggest that in hypercholesterolemia the observed increased blood vessel contractility might, at least in part, be due to the increased perinuclear Ca^{2+} signaling and reduced subplasmalemmal Ca^{2+} response.

Recent data indicate that in hypercholesterolemic animals, increased vascular O_2^- production occurs [18,19,33], which is thought to derive from membrane-bound NADH/NADPH oxidase on endothelial and smooth muscle cells [34–37]. In line with these data obtained in animals, we have found increased O_2^- production in human hypercholesterolemia. Our findings that a removal of the endothelium slightly reduced O_2^- release from the hypercholesterolemic vessels, while in healthy vessels O_2^- release tended to increase, is in agreement with previous reports in cholesterol-fed rabbits [18]. Nevertheless, unlike the report of Ohara et al., removal of the endothelium did not normalize increased O_2^- production in HC [18]. These data indicate that in human arteries hypercholesterolemia-associated increased O_2^- production is unlikely to be due to the endothelium. This is consistent with the findings in cholesterol-fed and atherosclerotic rabbits, where smooth muscle-derived O_2^- has been shown to attenuate endothelium-dependent relaxation [38,39].

Antioxidants, such as vitamins C and E, have been reported to normalize altered blood vessel function in hypercholesterolemia [16,40], thus indicating that the increased appearance of O_2^- is involved in vascular dysfunction during this disease. These conclusions are further supported by our findings that preincubation of normal smooth muscle cells with the O_2^--generating mixture, xanthine oxidase/hypoxanthine, mimicked the effect of hypercholesterolemia on smooth muscle Ca^{2+} signaling, while SOD diminished the effect of hypercholesterolemic serum on smooth muscle Ca^{2+} signaling. Previously, we have reported that O_2^- affects endothelial Ca^{2+} signaling, possibly by stimulation of tyrosine kinase [41]. Since tyrosine kinase has been shown to be involved in smooth muscle contractility [42–44], it needs to be further investigated, whether, besides the augmented elevation in $[Ca^{2+}]_{peri}$, O_2^--mediated tyrosine kinase activity, it contributes to the enhanced contractility of smooth muscle cells in hypercholesterolemia.

In conclusion our data suggest that, besides the endothelium, vascular smooth muscle cells need to be considered as potential targets of vascular dysfunction in human hypercholesterolemia. On the one hand, under hypercholesterolemia the vascular smooth muscle cells negatively interfere with the endothelium-derived relaxation by increased release of O_2^-. On the other hand, the observed changes in smooth muscle reactivity might be involved in the development of vascular complication and atherosclerosis in hypercholesterolemia.

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