Dihydropyridine calcium channel blockers inhibit non-esterified-fatty-acid-induced endothelial and rheological dysfunction

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Abstract
Circulating NEFAs (non-esterified fatty acids) from adipose tissue lipolysis lead to endothelial dysfunction and insulin resistance in patients with the metabolic syndrome or Type 2 diabetes mellitus. The aim of the present study was to test the hypothesis that DHP (dihydropyridine) CCBs (calcium channel blockers) prevent NEFA-induced endothelial and haemorheological dysfunction independently of their antihypertensive properties. Using a double-blind cross-over study design, nifedipine, amlodipine, diltiazem or placebo were administered to eight healthy subjects for 2 days before each study day. On the study days, the following were assessed before and after the infusion of lipid and heparin to raise serum NEFAs: endothelial function, by measuring FBF (forearm blood flow) responses to ACh (acetylcholine); leucocyte activation, by ex vivo measurement of plasma MPO (myeloperoxidase) levels, adherent leucocyte numbers and whole blood transit time through microchannels; and oxidative stress, by determining plasma levels of d-ROMs (derivatives of reactive oxygen metabolites). Effects of the CCBs on NF-κB (nuclear factor κB) p65 phosphorylation stimulated by NEFAs were assessed in cultured monocytic cells in vitro.

Elevated NEFAs reduced the responses to ACh and significantly increased whole blood transit time, adherent leucocyte numbers and d-ROMs. Nifedipine and amlodipine, but not diltiazem, prevented NEFA-induced endothelial dysfunction, leucocyte activation and enhancement of oxidative stress without affecting BP (blood pressure), whereas all these drugs prevented NEFA-induced p65 activation in vitro. These results suggest that DHP CCBs, independent of their antihypertensive properties in humans, prevent NEFA-induced endothelial and haemorheological dysfunction through inhibition of NEFA-induced leucocyte activation, although the sensitivity to drugs of leucocyte Ca2+ channels may differ among cells.

Key words: calcium channel blocker, endothelial function, leucocyte, non-esterified fatty acid (NEFA), oxidative stress

INTRODUCTION
Obesity and associated metabolic disorders constitute a serious threat for development of Type 2 diabetes and CVD (cardiovascular disease). Serum levels of NEFAs (non-esterified fatty acids), which are the products of adipose tissue lipolysis, correlate positively with amounts of visceral fat. NEFAs play an important role in development and progression of endothelial dysfunction and insulin resistance [1–3], and therefore the elevated levels may contribute to adverse cardiovascular events [4,5]. Experimental elevation of NEFAs by intravenous infusion of lipid and heparin significantly decreases the vasodilatory response to ACh (acetylcholine), as well as insulin sensitivity, in healthy subjects [2].

NEFA-induced endothelial dysfunction appears to be mediated, at least in part, through increased oxidative stress; previous studies by several investigators demonstrated that intra-arterial infusion of relatively high-dose vitamin C prevents this occurrence.

Abbreviations: ACh, acetylcholine; AngII, angiotensin II; BP, blood pressure; CCB, calcium channel blocker; DHP, dihydropyridine; d-ROM, derivatives of reactive oxygen metabolite; FBF, forearm blood flow; HBSS, Hanks balanced salt solution; HR, heart rate; MPO, myeloperoxidase; NEFA, non-esterified fatty acid; NF-κB, nuclear factor κB; ROS, reactive oxygen species; SNP, sodium nitroprusside.
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in humans [6,7], and also that NEFAs stimulate reduced NADPH oxidase and the PKC (protein kinase C) pathway to produce ROS (reactive oxygen species) in cultured human vascular cells [8,9].

On the other hand, NEFA-induced endothelial dysfunction may result from leucocyte activation [10], characterized by adhesion of leucocytes to endothelial cells and release of MPO (myeloperoxidase), ROS and cytokines. These vasoactive substances may collate to impair endothelial function. Indeed, we have shown that a bolus injection of heparin, which releases MPO from endothelial cells [11], effectively restores the attenuated response to ACh induced by NEFAs [10]. Considered together, these results indicate that leucocyte activation with oxidative stress may be a key cellular event that is relevant to endothelial dysfunction and further development of atherosclerosis.

CCBs (calcium channel blockers), widely used for treatment of patients with hypertension and angina pectoris, induce vasodilatation by blocking calcium ion influx into VSMCs (vascular smooth muscle cells). DHP (dihydropyridine) CCBs, unlike non-DHP CCBs, have antioxidant effects and increase NO bioavailability in vitro [12–15] and in vivo [16]. Experimental evidence suggests that functional DHP-sensitive Ca2+ channels are expressed in leucocytes [17]. A preliminary report showed that DHP CCB suppressed ROS production from the activated neutrophils [18–20]. Furthermore, previous clinical studies have demonstrated that DHP CCBs (1) effectively express endothelial function in patients with coronary artery disease, dyslipidaemia and/or hypertension, (2) prevent progression of atherosclerosis [21–24] and (3) in turn provide better cardiovascular outcome in patients with stable, symptomatic coronary disease, with or without hypertension [25,26]. Therefore this study was designed to test the hypothesis that DHP CCBs prevent NEFA-induced endothelial dysfunction and haemorheological disorder through antioxidative effects and inhibition of leucocyte activation.

**MATERIALS AND METHODS**

**Study participants**

Study participants consisted of 14 healthy, non-smoking and non-obese Japanese men, 20–35 years of age. All were normotensive with BMIs (body mass indices) of 21–24 kg/m²; no abnormalities were found on routine physical examinations or standard laboratory analyses after overnight fasting. None were taking any medications. The study protocol was approved by the ethics committee of the University of the Ryukyus, and written informed consent was obtained from all participants.

**Assessment of endothelial function before and after lipid/heparin infusions**

Participants fasted overnight and abstained from drinking beverages containing alcohol or caffeine for ≥12 h before the study. All experiments were performed in a quiet, temperature-controlled room (23–24 °C). Endothelial function was evaluated by the vasodilatation response, as described previously [7,10]. Briefly, the procedure consisted of infusions (through a 27-gauge needle inserted into the brachial artery of the non-dominant arm) of ACh (Daiichi Pharmaceutical) (in a stepwise fashion, from 50 nmol/min for 5 min to 100, 200 and 400 nmol/min for 3 min at each dose) or SNP (sodium nitroprusside) (3, 10 and 30 nmol/min), before and after the systemic intravenous infusion of normal saline (90 ml/h) and heparin (Shimizu Pharmaceutical) (0.3 unit/kg of body mass per min) (the control regimen) or lipid (Intralipid 20%; Fresenius Kabi) (90 ml/h) and heparin (0.3 unit/kg of body mass per min) for 1 or 2 h. FBF (forearm blood flow) was measured bilaterally in the resting supine position by venous occlusion plethysmography with strain gauges, as described previously [7,10,27].

**Measurement of plasma levels of NEFAs, MPO and serum levels of d-ROMs (derivatives of reactive oxygen metabolites)**

Plasma NEFA concentration was measured before and after 1 and 2 h lipid/heparin infusions in selected subjects (n = 6). Blood samples for NEFAs were placed in tubes containing 50 μl of paraoxon [diethyl PNPP (p-nitrophenyl phosphate)] (Sigma) diluted to 0.04 % in diethyl ether to prevent ex vivo lipolysis [28]. Samples were centrifuged immediately at 4 °C, and the plasma was collected and stored at −80 °C until it was assayed.

Plasma levels of MPO were determined using an ELISA kit (Bio Check) [10]. Levels of d-ROMs were assessed using the d-ROMs test kit (Dia cron) [29–31], which depends on Fenton-like reactions, leading to formation of lipid peroxy and alkoxy radicals that in turn react with a chromogenic substrate [29].

**Blood kinetics in an ex vivo microchannel capillary model**

A microchannel flow analyser (HR200; Kowa) was used as an ex vivo capillary model to assess whole blood rheology and leucocyte activity, as described previously [10,32–34]. Briefly, within 10 min after blood collection into a heparinized tube (5 % vol), the passage time for 0.1 ml of blood through microchannels (7854 parallel, 7 μm equivalent diameter and 30 μm long) under a constant suction pressure of −20 cm H2O was determined as a whole blood rheological parameter. Saline passage time was determined before each blood measurement, for calibration. The microscopic motion images of blood passing through the microchannels were monitored and stored on a computer system. In offline analysis, when 0.08–0.10 ml of blood had flowed out, five different still images were randomly selected and then the numbers of adhesive or plugging leucocytes on the microchannel terraces were counted. Adhesive leucocytes were defined as static leucocytes having clear surface borders on still images [10].

**Preparation of cultured cells**

THP-1 cells (a human monocytic cell line) were purchased from Health Science Research Resources Bank and maintained in RPMI 1640 medium (Gibco) supplemented with 5 % (v/v) complement-depleted FBS (fetal bovine serum) (Nichirei Biosciences) in a humidified CO2 incubator at 37 °C.
Clinical study protocol: effects of various CCBs on endothelial dysfunction, leucocyte activation and enhanced oxidative stress by NEFA provocation

Clinically relevant, intermediate doses of study drugs which had not affected BP (blood pressure) in preliminary experiments performed to explore BP-independent effects of CCBs on endothelial function and leucocyte activity, were selected for administration to eight normotensive men, according to the following protocol. Each subject received placebo, nifedipine CR 20 mg once daily, amiodipine 5 mg once daily or diltiazem retard 100 mg once daily for 2 days before each study day (study days 2–5) in a double-blind crossover design. There was a washout period of at least 14 days between study days. The order of drug administration was randomized. Endothelial function was assessed and blood samples were collected immediately before, and 1 and 2 h after, the initiation of systemic infusion of saline/heparin as control (study day 1) or lipid/heparin (study days 2–5). BP and pulse rate were non-invasively measured at time 0, and after 1 and 2 h of infusions (TM-2541R; A&D). Immediately after blood sampling and assessment of endothelial function at 2 h, a heparin bolus (70 units/kg of body mass) was injected to release vessel wall-immobilized MPO into the circulating bloodstream [10,11,35] and blood samples were collected. Newly released MPO from activated leucocytes by NEFA provocation was defined as 

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\Delta MPO = (\text{serum MPO after bolus injection of heparin following NEFA provocation}) - (\text{serum MPO after bolus injection of heparin during normal saline infusion}) \ [10].
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An additional experiment to explore the possible effects of a higher dose of diltiazem on leucocyte activation was performed as follows: six healthy males received 200 mg of either diltiazem or placebo for 2 days before the study day, and the effects of NEFA provocation on leucocyte activity were measured as described above.

Effects of various CCBs on NF-κB (nuclear factor κB) p65 phosphorylation stimulated by fatty acids in cultured monoyctic cells

A NEFA (Wako)–BSA (Sigma) solution was prepared as described previously [10]. The concentration of NEFAs in the product was determined using the NEFA assay kit. THP-1 cells were seeded into 10 cm culture dishes to a density of 5 × 10⁵ cells per a dish and incubated for 18 h with vehicle (DMSO 1/100 000) or one of the following CCBs: 20 ng/ml amiodipine, 30 ng/ml diltiazem or 50 ng/ml nifedipine (Sigma). The concentration of each CCB used was the expected peak concentration following the dose administered in the in vivo study. Either 2% (w/v) BSA in HBSS (Hanks balanced salt solution; Gibco) or fatty acid–BSA solutions (to achieve 0.05 mM fatty acid) was added to the dishes for 30 min. The cells were centrifuged and the pellets were washed with HBSS, denatured by 10% (w/v) TCA (trichloroacetic acid), and then lysed with a solution of 9 mol/l urea (Wako), 2% (v/v) Triton X-100 (ICN Biomedicals) and 1% (v/v) DTT (dithiothreitol) (MP Biomedicals). Next, a protein assay using Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories) was performed, and equal volumes of Western blotting SDS sample buffer (2 ×) was added to the lysates and boiled for 3 min. Equal amounts of protein were subjected to Western blotting using Mini-PROTEAN® TGX gels and Immun-Blots PVDF membrane (Bio-Rad Laboratories), followed by immunoblotting using anti-NF-κB p65 and anti-phospho-NF-κB p65 (Ser536) antibodies (Cell Signaling Technology). Reactive spots were detected by HRP (horseradish peroxidase)-linked secondary antibody and Hyperfilm ECL (enhanced chemiluminescence) (GE Healthcare). Resulting signals on the films were scanned into a computer and densities were analysed by Igor Pro (Wave-Metrics). The experiments were performed with each sample in duplicate, and repeated three times independently.

Statistical analysis

Data were statistically analysed using JMP 7.01 J software (SAS Institute). Continuous variables are described as means ± S.D. Probability values of P < 0.05 were considered to indicate statistically significant. Comparisons of Ach dose–response curves were made by repeated measures of ANOVA. Changes from the baseline on each experiment day in whole blood passage time through the microchannels, number of adhesive or plugging leucocytes in the microchannels and d-ROM levels at 1 and 2 h, as well as changes in plasma levels of MPO 15 min after heparin bolus, all following 2-h NEFA provocation, were analysed by one-way ANOVA followed by the Tukey–Kramer post-hoc test.

For in vitro experiments in culture cells, the Fisher’s PLSD (protected least-squares difference) test was applied to each data set (n = 6) for the determination of significant differences between pairs after performing ANOVA.

The power calculation for the present study was done according to the results of a previous pilot study (α = 0.05, 1 − β = 0.8). The expected difference in the change in whole blood passage time between placebo and DHP CCBs was 7 s, and the expected S.D. in a subject after NEFA exposure was 4.5 s. Using the equation:

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n = SD^2[Z_{α/2} + Z_{β}]^2/d^2 = (4.5)^2 \times 7.846/5^2 = 6.28
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it was determined that seven cases were needed to adequately power this study. Therefore eight healthy subjects were enrolled.

RESULTS

Baseline BP, HR (heart rate), FBF and haematocrit before lipid/heparin infusion did not differ significantly on the various experiment days (Table 1). After 1 and 2 h infusions of lipid/heparin, the plasma levels of NEFAs increased from 0.61 (0.29) to 1.70 (0.34) and 1.90 (0.44) mM respectively.

Effects of CCBs on NEFA-induced endothelial dysfunction

Elevation of NEFAs did not significantly affect BP, HR or baseline FBF. Dose–response curves for Ach during intravenous infusion of normal saline and saline/heparin were identical. Elevated NEFAs by infusion of lipid/heparin reduced the response of
resistance vessels to ACh, as reported previously [7,10]. Nifedipine CR and amlodipine prevented NEFA-induced endothelial dysfunction, although diltiazem retard did not (Figure 1). In addition, elevated NEFAs did not affect the FBF response to SNP following any experimental drug (results not shown).

Effects of CCBs on NEFA-induced leucocyte activation
Elevated NEFAs significantly prolonged the whole blood passage time in an \textit{ex vivo} capillary model. Pre-treatment with nifedipine CR or amlodipine significantly inhibited this prolongation at 2 h, although diltiazem retard did not (Figure 2a). Elevated NEFA significantly increased the number of adherent or plugging leukocytes, and pre-treatment with nifedipine CR or amlodipine significantly inhibited this response at 2 h, whereas diltiazem retard did not (Figures 2 and 3). Administration of the higher dose of diltiazem (200 mg/day) did not prevent NEFA-induced leucocyte activation (Figures 2c and 2d). MPO concentration after the bolus heparin injection following 2-h NEFA provocation + placebo pre-treatment was significantly higher than that after the heparin bolus following the control saline/heparin infusion (Figure 4). Pre-treatment with either nifedipine CR or amlodipine prevented the NEFA-induced increase in MPO, whereas diltiazem retard did not (Figure 4).
Non-esterified fatty acids and calcium channel blockers

Figure 2 Change from baseline, 1 and 2 h post-treatment, in (a) whole blood passage time and (b) number of adherent or plugging leucocytes, using an ex vivo capillary model

(a) Whole blood passage time was prolonged by lipid/heparin infusion; pre-treatment with nifedipine CR or amlodipine significantly inhibited this effect at 2 h, but diltiazem retard (DiltiazemR) did not. (b) The number of adherent or plugging leucocytes was increased by lipid/heparin infusion in a time-dependent manner; pre-treatment with nifedipine CR or amlodipine significantly inhibited this effect at 2 h. The administration of a higher dose of diltiazem at 200 mg/day did not prevent NEFA-induced leucocyte activation. (n = 6, c d). ∗P < 0.01 compared with NS/heparin, †P < 0.01 compared with placebo + NEFA, and ‡P < 0.05 compared with diltiazem R + NEFA, at each time by ANOVA with Tukey-Kramer post-hoc tests. FFA, NEFA.

The present double-blind crossover study demonstrated that DHP CCBs (nifedipine CR and amlodipine), but not a non-DHP CCB (diltiazem retard), prevented NEFA-induced leucocyte activation and oxidative stress. These effects consequently prevented endothelial and haemorheological dysfunction after the lipid/heparin infusion partly through inhibition of adhesion of leucocytes and MPO release from activated leucocytes, which impairs NO availability and adversely modulates vascular function. Our results may provide one mechanistic explanation for improvement of endothelial function after nifedipine, independently of BP effects, in patients with dyslipidaemia [22].

One might be surprised to find that DHP CCBs prevented NEFA-induced leucocyte activation, as it has long been thought that voltage-operated L-type Ca\(^{2+}\) channels are expressed only in excitable cells, and not in unexcitable cells such as leucocytes. We therefore can plausibly assume that antioxidative effects of DHP CCBs indirectly prevented NEFA-induced leucocyte activation [36]. Indeed, we clearly demonstrated that d-ROM concentrations, which reflect the serum hydroperoxide levels [29–31], were significantly elevated after the 2-h NEFA provocation and that administration of DHP CCBs significantly inhibited this effect, consistent with the results from previous in vitro and in vivo experiments [12–16,19]. Indirect protection against leucocyte activation might result from up-regulated NO bioavailability [37,38] by DHP CCBs through VEGF (vascular endothelial growth factor) from smooth muscle cells [14,39–41], although endothelial cells do not possess voltage-operated L-type Ca\(^{2+}\) channels.

Previous evidence suggests the existence of functional DHP-sensitive channels in a variety of non-excitable cells, including various haematopoietic cells such as B cells [42,43], neutrophils [17] and T-cells [44]. Given that Ca\(^{2+}\) signalling is relevant to a number of leucocyte activities our present observation implies that DHP CCBs may directly inhibit NEFA-induced leucocyte activation through DHP-sensitive channels. In fact, Rosales and Brown [17] showed that nifedipine inhibited Ca\(^{2+}\) release from intracellular stores in neutrophils. More interestingly, nifedipine reportedly suppressed the production of ROS in activated neutrophils partly through inhibition of the rise in intracellular Ca\(^{2+}\) [18,19] or a mechanism other than antagonizing Ca\(^{2+}\) influx [20]. Amlodipine also reverses the elevation of intracellular Ca\(^{2+}\) and impairment of phagocytosis in neutrophils from patients with uncontrolled Type 2 diabetes mellitus [45]. By considering these
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Figure 3 Representative microphotographs of adherent or plugging leucocytes in whole blood after treatment with CCB using an ex vivo capillary model (microchannel flow analyser). Increased serum NEFAs significantly increased the number of adherent or plugging leucocytes (arrows); 2-day pre-treatment with (b) nifedipine CR or (c) amlodipine significantly inhibited this effect, but (d) diltiazem R did not.

Figure 4 Change in plasma levels of MPO 15 min after heparin bolus injection (70 units/kg of body mass) following 2-h NEFA provoke with or without CCB pre-treatment. MPO levels were significantly increased by 2-h NEFA provoke + placebo. Pre-treatment with either nifedipine CR or amlodipine prevented this effect; however, diltiazem retard did not. *P < 0.05 compared with NS (normal saline)/heparin, and †P < 0.05 compared with NEFA + placebo. FFA, NEFAs.

Observations together, it can be hypothesized that nifedipine and amlodipine may directly inhibit NEFA-induced leucocyte activation through DHP-sensitive Ca\(^{2+}\) channels and, as a consequence, could prevent NEFA-induced endothelial and haemorheological dysfunction. It should be noted, however, that not only DHP CCB but also diltiazem might directly inhibit leucocyte activation through L-type Ca\(^{2+}\) channels [17,19,20,42]. In fact, unlike the current ex vivo data, we clearly demonstrated the similar inhibitory effects of all CCBs on NEFA-induced NF-κB p65 activation in THP-1 cells, which is consistent with a previous report [46].

There is inconsistency among the results of previous studies regarding the inhibitory effects of DHP CCBs and diltiazem on activation of neutrophils. Some revealed similar effects [17,19,20] and others did not [18,47]. Differences in experimental models used (e.g. in vitro compared with ex vivo), types of cells used and leucocyte activation methods employed may account for these inconsistencies.

Recently we showed that NEFA-induced leucocyte activation is, at least in part, angiotensin-dependent [10]. As AngII (angiotensin II) promotes cytosolic Ca\(^{2+}\) accumulation from intracellular Ca\(^{2+}\) stores and Ca\(^{2+}\) influx in activated neutrophils [48], our present results could partly be explained by DHP-CCB inhibition of Ca\(^{2+}\) signalling in leucocytes. Alternatively, DHP CCBs could indirectly inhibit AngII-dependent leucocyte activation through suppression of the oxidative stress that acts as a critical mediator of AngII signalling in human neutrophils [49].

Limitations
First, the effects of only one dosage level of each CCB for 2 days duration were studied. The dose–effect relationship for each CCB was not demonstrated and compared with that of the others. There were differences in the anti-leucocyte and antioxidant effects among the three CCBs used, which may be because of the potency of the drugs [50] or be due to differences in their pharmacokinetics. Considering the longer half-life of amlodipine, oral administration for 2 days may not have been sufficient for the achievement of an adequate steady-state concentration to produce significant effects on leucocyte activation and oxidative stress. Therefore the effects of amlodipine on endothelial function, leucocyte activity and oxidative stress might have been underestimated.

Secondly, DHP CCBs may prevent NEFA-induced leucocyte activation directly and indirectly through reduction in oxidative stress. The question of which was the main factor working against leucocyte activation cannot clearly be answered by the results of this study, because activated leucocytes are the major source of ROS, and ROS causes leucocyte activation.

In conclusion, the results of this study suggest that DHP CCBs prevent NEFA-induced endothelial and haemorheological dysfunction through an antioxidative effect and direct inhibition of leucocyte activation by Ca\(^{2+}\) signalling pathways. Further investigations into the effects of long-term NEFA provocation are warranted to extrapolate the present results to obese patients, who have chronically elevated NEFA plasma levels.

CLINICAL PERSPECTIVES

- NEFAs play an important role in the development and progression of endothelial dysfunction and, therefore, they may contribute to cardiovascular events. Dihydropyridine calcium channel blockers (DHP CCBs) may ameliorate such adverse effects of NEFAs through pleotropic effects other than blood pressure reduction.
- In the present study, we have investigated the effects of DHP CCBs and show that they prevent NEFA-induced endothelial and haemorheological dysfunction through an antioxidative effect and the direct inhibition of leucocyte activation.
Figure 5  Changes from baseline in plasma levels of d-ROMs (a measure of oxidative stress) 1 and 2 h post-treatment on each study day
Levels were increased time-dependently by lipid/heparin infusion (black bars). Pre-treatment with nifedipine CR significantly, and with amlodipine moderately, inhibited this effect at 2 h, but diltiazem retard did not. 1 U.CARR = 0.8 mg/l H₂O₂.

Figure 6  CCBs prevent activation of NF-κB p65 by fatty acids in monocytic cells
Representative blots with quantification in the bar graph (normalized ratio of pho-p65/total p65, n = 6, means ± S.D.) are shown. THP-1 cells were pre-treated with DMSO (CCB vehicle) or amlodipine 20 ng/ml (Aml) or diltiazem 30 ng/ml (Dilt) or nifedipine 50 ng/ml (Nif) for 18 h prior to application of NEFAs at a final concentration of 0.05 mM. *P < 0.05 compared with DMSO–NEFA group.

The results of the present study may support use of DHP CCBs for their anti-atherosclerotic actions in patients with visceral fat obesity and in those with established diabetes, whose NEFA levels are assumed to be high.

AUTHOR CONTRIBUTION
Takanori Yasu, Ken Yamakawa and Shinichiro Ueda contributed to the conception and design of the study, the analysis and the interpretation of the data and writing of the paper. Takanori Yasu and Mayumi Kobayashi performed in vivo and ex vivo experiments. Akiko Mutoh performed in vitro experiments and contributed to the interpretation of the data and writing of the paper. Shin-ichi Momomura contributed to the measurement of d-ROMs and the interpretation of the data.

ACKNOWLEDGEMENT
We thank Sachimi Jimbo for technical assistance in measurement of d-ROM levels.

FUNDING
This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan [grant numbers 16590439 (to S.U.) and 20590542 (to K.Y.)] and from the Vehicle Racing Commemorative Foundation (to S.M. and T.Y.).

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The Authors

Journal compilation C T. Yasu and others


Non-esterified fatty acids and calcium channel blockers


Received 12 June 2012/25 March 2013; accepted 27 March 2013
Published as Immediate Publication 27 March 2013, doi: 10.1042/CS20120311

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