Chronic exposure to high fatty acids impedes receptor agonist-induced nitric oxide production and increments of cytosolic Ca$^{2+}$ levels in endothelial cells

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Abstract

Dyslipidemia is a common metabolic disorder in diabetes. Nitric oxide (NO) production from endothelium plays the primary role in endothelium-mediated vascular relaxation and other endothelial functions. Therefore, we investigated the effects of elevated free fatty acids (FFA) on the stimulation of NO production by phospholipase C (PLC)-activating receptor agonists (potent physiological endothelium-dependent vasodilators) and defined the possible alterations of signaling pathways implicated in this scenario. Exposure of bovine aortic endothelial cells (BAECs) to high concentrations of a mixture of fatty acids (oleate and palmitate) for 5 or 10 days significantly reduced NO production evoked by receptor agonists (bradykinin or ATP) in a time- and dose-dependent manner. Such defects were not associated with alterations of either endothelial NO synthase mass or inositol phospholipid contents but were probably due to reduced elevations of intracellular free Ca$^{2+}$ levels ($[\text{Ca}^{2+}]_i$) under these conditions. Exposure of BAECs to FFA significantly attenuated agonist-induced $[\text{Ca}^{2+}]_i$ increases by up to 54% in a dose- and time-dependent manner. Moreover, bradykinin receptor affinity on the cell surface was significantly decreased by high concentrations of FFA. The morphology of BAECs was altered after 10-day culture with high FFA. Co-culture with protein kinase C (PKC) inhibitors or antioxidants was able to reverse the impairments of receptor agonist-induced NO production and $[\text{Ca}^{2+}]_i$ rises as well as the alteration of receptor affinity in BAECs exposed to FFA. These data indicate that chronic exposure to high FFA reduces NO generation in endothelial cells probably by impairing PLC-mediated Ca$^{2+}$ signaling pathway through activation of PKC and excess generation of oxidants.

Journal of Molecular Endocrinology (2011) 47, 315–326

Introduction

Endothelium plays an important role in the regulation of vascular tone and other functions via the synthesis and release of vascular modulators. Nitric oxide (NO), a gas molecule and potent vasodilator, has received the most interest in the study of endothelial dysfunction since it was recognized in 1987 (Moncada et al. 1991). In endothelial cells (ECs), NO is synthesized by endothelial NO synthase (eNOS) and eNOS activity is regulated mainly by a change in cytosolic free Ca$^{2+}$ concentrations ($[\text{Ca}^{2+}]_i$). Phospholipase C (PLC)-activating receptor agonists released from nerves (such as acetylcholine and ATP) or circulated from other tissues (e.g. bradykinin) can increase eNOS activity in this manner to dilate blood vessels under physiological conditions (Mombouli & Vanhoutte 1995). The NO released from ECs diffuses to and activates guanylate cyclase in the adjacent vascular smooth muscle cells, resulting in an increase in cGMP production, which triggers a cascade of reactions leading to relaxation of the blood vessel (Moncada et al. 1988, Kuchan & Frangos 1994). In addition, NO plays an important role in inhibiting inflammation and atherosclerosis as it reduces the adhesion and interaction of white cells with endothelium. Thus, an alteration in NO production and/or degradation might make a significant contribution to the deregulation of vascular tone and pathogenesis of various diabetic vascular diseases (Moncada et al. 1988, Capellini et al. 2010, Ding & Triggle 2010, Triggle & Ding 2010).

More data have revealed that endothelium-dependent vascular relaxation, which is an early indicator of endothelium dysfunction (Durante et al. 1988, McVeigh et al. 1992, Schiekofer et al. 2000, Ding & Triggle 2010), is impaired in various types of blood vessels in experimental diabetic animals, as well as in both type 1 and type 2 diabetic patients. It is known that, in addition to increased plasma glucose levels, type 2 diabetes is associated with increased plasma levels of free fatty acids (FFAs) resulting from lack of appropriate suppression of adipocyte lipolysis due to insulin...
resistance and deficiency (Reaven 1988, Imrie et al. 2010). Furthermore, circulating levels of FFAs are usually elevated at the time of diagnosis, and also indeed in the pre-diabetic phase that lasts for a long period before hyperglycemia occurs while endothelial dysfunction can be detected (Reaven et al. 1988, Hamilton et al. 2007, Rask-Madsen & King 2007, Mugabo et al. 2011). Some studies have reported a modulating effect of FFAs on endothelium-dependent NO production and thereby on endothelial function in diabetic patients (Davda et al. 1995, Steinberg et al. 2000, Imrie et al. 2010). Moreover, raising FFAs in normal human volunteers to the levels observed in obese hypertensive patients impairs endothelium-dependent vasodilation (Davda et al. 1994, Tripathy et al. 2003). In vitro studies showed that oleic acid impedes eNOS activity in cultured ECs and impairs endothelium-dependent relaxation in vascular rings (Davda et al. 1995, Esenabhalu et al. 2003). These observations increase the possibility that FFAs may have significant effects on the cardiovascular system and contribute to functional and structural vascular changes in diabetes.

However, the mechanism underlying the modulating effect of FFAs on endothelial dysfunction in diabetes has been poorly investigated and it is unclear how FFAs cause a reduction of eNOS activity. The results from short-term cultured ECs are conflicting. One study demonstrated that 10-min overload with FFAs inhibited Ca\(^{2+}\) mobilization in ECs (Kuroda et al. 2001). In contrast, another study observed that overall intracellularly stored Ca\(^{2+}\) and intracellular Ca\(^{2+}\) release were enhanced after ECs were exposed to FFAs for 4 h, while the attenuated eNOS activity was associated with O\(_2\)\(^-\) release (Esenabhalu et al. 2003).

To date, there are no reports on the chronic effect of FFAs on cultured ECs, which is more relevant to the development of diabetes-related cardiovascular complications and endothelial dysfunction. Thus, this study was designed to elucidate the long-term effects of FFAs on the signaling pathway for NO production in ECs.

**Materials and methods**

**Culture and pre-treatment of bovine aortic ECs**

Bovine aortic ECs (BAECs) up to passage 13 were first grown to near confluence in MCDB 131 containing 10% FCS and media were replaced every 48 h. The cells were then cultured with control (1% BSA) or various concentrations (0.25, 0.5, or 1.0 mmol/l) of FFAs (a mixture of olate and palmitate, 2:1 (w/w)) (Hirose et al. 1996) for 2, 5, or 10 days with concomitant lowering of the serum concentration in the medium to 2% to keep the cells in the quiescent state; media were replaced every 24 h during these periods. Other test agents such as protein kinase inhibitors were also included during the culture periods as specified. However, FFA and other test agents were not included in all buffer solutions used in the experiments described below, in order to avoid possible acute effects.

**Measurement of NO production**

BAEC suspensions were obtained by mild trypsinization. The harvested BAECs were centrifuged and resuspended in DMEM containing 2% FCS. After incubation in a spinner for 2.5 h at 37 °C, the cells were loaded with 2 nmol/l 4,5-diaminofluorescein diacetate (DAF-2/DA, a membrane-permeable fluorescent NO probe) at 37 °C for 30 min in darkness (Nakatsubo et al. 1998). DAF-2-loaded cells were resuspended in DMEM in a density of 2.5×10\(^5\) cells/ml. Aliquots of cells (200 μl) were added into 96-well black plate and kept at 37 °C for 15 min. Then, stimuli were added and incubation was lasting for up to 3–6 h at 37 °C. The fluorescence generated by NO production was monitored by a fluorescence plate reader at excitation and emission wavelengths of 485 and 515 nm respectively (Tang & Li 2004).

**Measurement of [Ca\(^{2+}\)]\(_i\)**

[Ca\(^{2+}\)]\(_i\) was measured using the fura-2 technique as described previously (Li et al. 1991, Tang & Li 2004). Briefly, cultured BAECs were harvested, centrifuged, and resuspended in DMEM medium containing 2% FCS. After incubation in a spinner for 2.5 h at 37 °C, the cells were loaded with 1 μmol/l fura-2/AM for 30 min. BAECs were then centrifuged, washed twice, and resuspended (final density of 1.25×10\(^6\) cells/ml) in a mixture (1:2, v/v) of HEPES (20 mmol/l)-buffered Krebs–Ringer solution (126 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l CaCl\(_2\), 1 mmol/l MgSO\(_4\), pH 7.4) and DMEM medium with 2% FCS. Aliquots of cell suspensions were centrifuged and added to a cuvette containing 2 ml HEPES-buffered KRB solution. The fura-2-generated fluorescence was monitored with a spectrophuorometer (Perkin–Elmer LS-50B; Perkin–Elmer, Waltham, MA, USA) and recorded by ratio fluorometry with emission wavelength of 515 nm at alternate 340/380 nm excitation wavelengths. The fluorescence signals were calibrated into [Ca\(^{2+}\)]\(_i\) values by an equation as described previously (Li et al. 1991).

**Assessment of bradykinin binding to its receptor**

Binding of bradykinin to its receptors in BAECs was assessed by radioligand saturation binding assay as described (Huang & Gibson 1996, Tang & Li 2004).

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**Journal of Molecular Endocrinology (2011) 47, 315–326**

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Cells were grown in 6-well culture plates with control or various concentrations of FFAs in the absence or presence of protein kinase C (PKC) inhibitors. Cells were washed twice with ice-cold PBS and rinsed with 2 ml binding buffer (Tang & Li 2004). Afterward, cells were equilibrated in chilled binding buffer for 15 min on ice and the medium was replaced by 1·5 ml binding buffer containing 0·5 nmol/l [3H]bradykinin (90–120 Ci/mmol; Amersham Biosciences) with different concentrations of unlabeled bradykinin (0·1–50 nmol/l) for 2 h at 4°C. For assessing non-specific binding, cells were incubated in the presence of 5 μmol/l cold bradykinin. The medium was then removed and cells were rapidly rinsed four times with a total of 20 ml ice-cold rinsing buffer (Tang & Li 2004). Subsequently, cells were detached by trypsinization and transferred to scintillation vials. After adding scintillation cocktail and mixing thoroughly, radioactivity in the samples was determined by a β-counter. The number and affinity of bradykinin receptors were calculated by Scatchard plot and analyzed as described elsewhere (Limbird 1996).

Determination of eNOS and iNOS by western blotting

BAECs were cultured at different concentrations of FFAs. After two washes with cold PBS, cells were extracted in homogenizing buffer consisting of 50 mmol/l Tris (pH 8·0), 150 mmol/l NaCl, 0·02% NaN₃, 100 μg/ml phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100. Protein content was determined by a colorimetric assay (Bio-Rad Protein assay). Equal amounts of lysates (40 μg protein) were denatured, separated by SDS–PAGE, and transferred onto nitrocellulose membranes. After blocking in 5% nonfat milk, the membranes were hybridized for 90 min in Tris-buffered saline (TBS; 100 mmol/l Tris/HCl and 0·9% NaCl) with anti-eNOS or anti-iNOS monoclonal antibody (1:1000 dilution). The membranes were washed and incubated in TBS buffer with goat anti-mouse IgG (1:2000 dilution). Following the enhanced chemiluminescence development (Pierce, Rockford, IL, USA), the membranes were subjected to autoradiography and were then analyzed by densitometry.

Measurement of cellular inositol phospholipids by thin layer chromatography

BAECs were seeded in 75 cm² flasks and cultured with various concentrations of FFAs for 10 days. On the day of experiment, cells were incubated in a 4 ml phosphate-free DMEM medium containing 0·37 mCi/ml 32P₂O₅ for 2 h. After washing, cells were harvested by trypsinization and transferred to glass tubes. Cell pellets from centrifugation were subject to repeated extraction with CHCl₃–CHOH–2·4 mol/l HCl (1·5:1·5:1·5; v/v/v) to obtain total cellular lipids (Vanhoutte 1995). Phospholipid standards and extracted lipid samples were loaded on pre-activated TLC plates, which were developed into two successively different solvent systems (Medh & Weigel 1989). The 32P-labeled lipids were visualized by autoradiography and the spots corresponding to phosphatidylinositol-4,5-bisphosphate (PIP₂), PIP, and PI were scraped into vials. After mixing well with scintillation cocktail, the radioactivity was determined by a β-counter.

Statistical analysis

Results are expressed as mean ± S.E.M.; n stands for the number of experiments conducted. Statistical analyses of the data were performed using unpaired two-tailed t-test or ANOVA with Bonferroni post hoc correction.

Results

Chronic FFA overload impairs receptor agonist-induced NO formation

PLC-activating receptor agonists such as bradykinin and ATP are potent vasodilators by increasing eNOS activity through elevation of \([\text{Ca}^{2+}]_i\) in ECs (Mombouli & Vanhoutte 1995). The receptor agonist bradykinin (1 μmol/l) induced an almost linear increase in NO production over 6 h incubation in BAECs pre-cultured at control (1% BSA) condition or low (0·25 mmol/l) FFAs (Fig. 1A and B). Such bradykinin-stimulated NO generation could be blocked by 100 μmol/l N⁵-nitro-L-arginine methylester (l-NAME), an inhibitor of eNOS (not shown). However, chronic exposure of BAECs to higher concentrations (0·5 and 1·0 mmol/l) of FFAs reduced bradykinin-induced NO production in a time- and concentration-dependent manner (Fig. 1A and B). For instance, the NO formation over 120 min stimulation was significantly reduced by 28 and 40% after 5-day culture at 0·5 and 1·0 mmol/l FFAs respectively (Fig. 1A). Further reduction of bradykinin-stimulated NO production over the same period (by 34 and 49% respectively) occurred following 10-day culture with high FFAs (Fig. 1B). Similar inhibitory effects were also observed on NO production induced by purinergic stimulation with 10 μmol/l ATP (also a PLC-activator) in another type of ECs, human vein ECs (Supplementary Figure 1, see section on supplementary data given at the end of this article). In contrast, overload of FFAs did not alter ionomycin (a Ca²⁺-ionophore)-induced NO production in BAECs (Fig. 1C). However, 2-day culture with high fatty acids (0·5 or 1·0 mmol/l) was unable to alter bradykinin-induced NO production (data not shown).
Sustained culture in high FFAs has no effect on NOS protein mass

Examination by western blotting did not reveal significant alteration in eNOS mass in BAECs cultured with FFAs for either 5 or 10 days (Supplementary Figure 2, see section on supplementary data given at the end of this article), suggesting that the activity, rather than the mass, of eNOS in BAECs might be affected by FFA treatment. Furthermore, culture with FFAs did not induce BAECs to express iNOS (data not shown), another isoform of NOS that can produce NO via an inducible pathway.

Chronic high FFA culture selectively reduces receptor agonist-induced [Ca^{2+}]_{i} rises

In the control BAECs, stimulation by 1 μmol/l bradykinin resulted in a rapid increase in [Ca^{2+}]_{i} from a baseline of 122 ± 34 nmol/l to a peak of 544 ± 47 nmol/l (the average of [Ca^{2+}]_{i} rises during the initial 30 s). Culture with FFAs had no effect on basal [Ca^{2+}]_{i} levels. In addition, pre-treatment with low (0.25 mmol/l) FFAs for 5 or 10 days did not significantly alter bradykinin-induced [Ca^{2+}]_{i} increments (data not shown). However, the ability of bradykinin to elevate [Ca^{2+}]_{i} was markedly reduced in cells pre-cultured for 5 days with higher concentrations of FFAs; the increments in peak [Ca^{2+}]_{i} were decreased by 21 and 34% at 0.5 and 1.0 mmol/l FFAs respectively (Fig. 2A and C). Exposure to high concentrations of FFAs for 10 days further reduced the effect of bradykinin; the peak [Ca^{2+}]_{i} increments were decreased by 34 and 54% after culture at 0.5 and 1.0 mmol/l of FFAs respectively (Fig. 2B and C). Similarly, high FFAs also inhibited [Ca^{2+}]_{i} rises induced by another PLC-activating agonist, 10 μmol/l ATP (data not shown).
In contrast, \([\text{Ca}^{2+}]_i\) elevations evoked either by thapsigargin (1 \(\mu\text{mol/l}\)), a specific inhibitor of the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase resulting in \(\text{Ca}^{2+}\) release intracellularly, or by ionomycin (1 \(\mu\text{mol/l}\)), a \(\text{Ca}^{2+}\) ionophore, were not significantly affected by 10-day FFA culture (Supplementary Figure 3A and B, see section on supplementary data given at the end of this article). These results implied that the releasable \(\text{Ca}^{2+}\) stores in BAECs were not affected by FFAs. Furthermore, this might also explain why culture with high concentrations of FFAs did not interfere with ionomycin-stimulated NO production (cf. Fig. 1C).

**Culture with high FFAs inhibits bradykinin-evoked \(\text{Ca}^{2+}\) mobilization and \(\text{Ca}^{2+}\) influx**

Neither 2-day culture with high FFAs nor up to 10-day culture with low (0.25 mmol/l) FFAs affected bradykinin-induced \(\text{Ca}^{2+}\) mobilization and \(\text{Ca}^{2+}\) influx (Supplementary Table 1, see section on supplementary data given at the end of this article). However, after 5-day culture with 0.5 or 1.0 mmol/l FFAs, bradykinin-induced \(\text{Ca}^{2+}\) mobilization was significantly reduced by 19 and 24\% \((P<0.01)\) while the \(\text{Ca}^{2+}\) influx was inhibited by 17 and 30\% \((P<0.01)\) respectively (Fig. 2D and Supplementary Table 1). Further reduction of bradykinin-induced \(\text{Ca}^{2+}\) mobilization and \(\text{Ca}^{2+}\) influx was observed after 10-day culture under these conditions; the former was diminished by 24 and 32\% \((P<0.01)\) and the latter was blunted by 35 and 57\% \((P<0.01)\) respectively (Fig. 2E and Supplementary Table 1). However, such an impairment by high FFAs was apparently not due to an alteration of intracellular \(\text{Ca}^{2+}\) stores, since the ability of thapsigargin to promote both \(\text{Ca}^{2+}\) mobilization and \(\text{Ca}^{2+}\) influx was not affected (Supplementary Table 2, see section on supplementary data given at the end of this article).

**Figure 2** Effect of FFAs on bradykinin-induced increases of \([\text{Ca}^{2+}]_i\) in BAECs. Bradykinin (BK; 1 \(\mu\text{mol/l}\))-induced increases in \([\text{Ca}^{2+}]_i\) in BAECs cultured for 5 days (A) or 10 days (B) at control (1% BSA), normal (0.25 mmol/l), or high (0.5 or 1.0 mmol/l) concentrations of FFAs in cell suspensions were determined using the fluorescent \(\text{Ca}^{2+}\) probe fura-2. Each trace is the superimposition of six experiments. (C) The average increases in \([\text{Ca}^{2+}]_i\) during the initial 30 s of bradykinin stimulation. Bar values represent means \pm S.E.M. \(** P<0.01\) vs control. Open bars, 5 days of culture; close bars, 10 days of culture. (D and E) Bradykinin (BK, 1 \(\mu\text{mol/l}\))-induced \(\text{Ca}^{2+}\) mobilization from intracellular stores was determined by chelating \(\text{Ca}^{2+}\) in the extracellular buffer to nominal \(\text{Ca}^{2+}\)-free levels by adding 3 mmol/l EGTA, whereas bradykinin-promoted \(\text{Ca}^{2+}\) influx was assessed by restoration of extracellular free \(\text{Ca}^{2+}\) to the normal level by adding 3 mmol/l \(\text{Ca}^{2+}\) to the solution. Cells were cultured with control (1% BSA), normal (0.25 mmol/l), or high (0.5 or 1.0 mmol/l) concentrations of FFAs for 5 days (D) or 10 days (E). Each trace is the representative of seven observations.
FFAs does not alter the content of inositol phospholipids in BAECs

One possibility for the reduced [Ca^{2+}]_{i} responses to the stimulation by receptor agonists in high FFA-cultured cells might be a decrease in the production of inositol 1,4,5-triphosphate (IP3), since bradykinin-induced Ca^{2+} mobilization was diminished. Such an effect could be due to an inhibition of PLC and/or to a reduction of its substrate, PIP2. However, culturing BAECs for 10 days with 0.25, 0.5, or 1.0 mmol/l FFAs did not cause significant changes in the contents of any of the three inositol phospholipids: PI, PIP, and PIP2 as measured by TLC (Fig. 3).

High FFAs decrease the affinity of bradykinin receptor in BAECs

Performance of radioligand binding assay detected only one type of bradykinin receptors in BAECs since all plots fitted a single receptor model (Fig. 4). The receptor had a $K_d$ of $0.83 \pm 0.08$ nmol/l, the total number ($B_{max}$) of receptors was $365 \pm 22$ fmol/mg protein. Culture with 0.25 mmol/l FFAs for 10 days did not alter either the receptor number (350 $\pm 17$ fmol/mg protein) or the receptor affinity (0.84 $\pm 0.09$ nmol/l). In contrast, 10-day culture with higher concentrations of FFAs significantly reduced the affinity of bradykinin receptors, $K_d = 1.21 \pm 0.1$ and 1.46 $\pm 0.17$ nmol/l at 0.5 and 1.0 mmol/l FFAs respectively ($P<0.01$). However, the number of bradykinin receptors ($B_{max}$) was not affected (350 $\pm 15$ and 340 $\pm 24$ nmol/l at 0.5 and 1.0 mmol/l of FFAs respectively; $P>0.05$) under these conditions.

High FFAs alter the morphology of BAECs

The morphology of ECs was modified by FFAs (Fig. 5). Control BAECs grown on culture plates were flat and the cell boundary was ambiguous (Fig. 5A). When cultured with 0.25 mmol/l FFAs for 10 days, the cell boundary became clear and the cell density was higher (Fig. 5B), suggesting that a physiological concentration of FFAs might promote the proliferation of ECs. However, higher concentration of FFAs, such as 0.5 and 1.0 mmol/l, caused the cells to lose their normal shiny and smooth surface, which became jagged and coarse (Fig. 5C and D). Cells cultured with 1.0 mmol/l FFAs were also flattened and their boundaries were unrecognizable (Fig. 5D).

PKC inhibitors and antioxidants reverse impairments due to high FFA culture

To study the possibility that PKC and oxidants are involved in the above observed alterations of agonist-evoked responses by long-term culture of FFAs, we co-cultured BAECs with several cell-permeable PKC inhibitors and antioxidants (Table 1). After 5-day co-culture, the impairment by 0.5 and 1.0 mmol/l FFAs on bradykinin-evoked NO production over 90 min was reversed by 78 and 74%, respectively, by bisindolylmaleimide-I (10 $\mu$mol/l; a general PKC inhibitor). Similar alleviating effects (by 67 and 71% respectively) were observed when GO6976 (20 nmol/l; a selective inhibitor of Ca^{2+}-dependent PKC-$\beta$ isoforms) was co-cultured. In addition, $\alpha$-tocopherol (100 $\mu$mol/l; an antioxidant but also capable of serving as a general PKC inhibitor through preventing diacylglycerol formation presumably by activating diacylglycerol kinase (Meier & King 2000)) also attenuated the inhibitory

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**Figure 3** Fatty acid overload has no effect on PI, PIP, and PIP$_2$ in BAECs. BAECs were cultured with control (1% BSA), normal (0-25 mmol/l), or high FFAs (0.5 and 1.0 mmol/l) for 10 days. After labeling with $^{32}$P, cell lipids were extracted, separated by thin layer chromatography, visualized by autoradiography, and quantified by $\beta$-ray counting. Values are mean $\pm$ S.E.M. of four independent experiments. There is no significant statistical difference by $t$-test.

**Figure 4** Effect of FFAs on the affinity of bradykinin receptor in BAECs. Scatchard plot analyses were performed by a nonlinear curve-fitting program (Sigma Plot; SPSS, Chicago, IL, USA) to determine the dissociation constants of bradykinin receptor. Data are from four independent observations. The plot is expressed as bound/free on the ordinate vs bound on the abscissa, where bound means concentration of ligand–receptor complex and free means concentration of free radioligand at equilibrium. These data were analyzed by ANOVA with Bonferroni post hoc correction.

**Figure 5** BAECs. BAECs were cultured with control (1% BSA), normal (0.25 mmol/l), or high FFAs (0.5 and 1.0 mmol/l) for 10 days. After labeling with $^{32}$P, cell lipids were extracted, separated by thin layer chromatography, visualized by autoradiography, and quantified by $\beta$-ray counting. Values are mean $\pm$ S.E.M. of four independent experiments. There is no significant statistical difference by $t$-test.
BAECs. In addition, longer (10-day) co-culture with these PKC inhibitors and antioxidants also reduced the impairment of NO production due to 0.5 or 1.0 mmol/l FFAs (bisindolylmaleimide-I (76 and 75%), GÖ (66 and 75%), α-α-tocopherol (84 and 81%), and N-acetyl-L-cysteine (82 and 80%) (Table 1).

The observed reduction of agonist-evoked [Ca\(^{2+}\)]\(_i\) rises by high concentrations of FFAs could also be largely reversed by PKC inhibitors and antioxidants (Fig. 6 and Table 2). Five-day co-culture with bisindolylmaleimide-I reversed the inhibitory effect of 0.5 and 1.0 mmol/l FFAs by 83 and 72% respectively. GÖ 6976 also attenuated the adverse effect of FFAs by 83 and 71% under the same conditions. Likewise, α-α-tocopherol was able to overturn the reduced [Ca\(^{2+}\)]\(_i\), rise by 73 and 71% due to 0.5 and 1.0 mmol/l FFAs respectively. N-acetyl-L-cysteine also ameliorated the impairment by 80 and 71%. Similar degrees of reversal on the reduced [Ca\(^{2+}\)]\(_i\), responses to bradykinin by 10-day culture of 0.5 and 1.0 mmol/l FFAs were also observed in the presence of either PKC inhibitors or antioxidants: 80 and 75% by bisindolylmaleimide-I, 68 and 73% by GÖ 6976, 75 and 79% by α-α-tocopherol, and 85 and 80% by N-acetyl-L-cysteine respectively (Table 2).

Furthermore, co-culture of bisindolylmaleimide-I or α-α-tocopherol was able to reverse the reduced affinity of bradykinin receptor in BAECs resulting from 10-day culture with high FFAs (Table 3). Bisindolylmaleimide-I restored the receptor affinity by 87 and 91% at 0.5 and 1.0 mmol/l FFAs respectively. α-α-tocopherol restored the affinity of bradykinin receptor by 91 and 92% accordingly. Finally, the morphology of BAECs returned to normal by large after co-culture with α-α-tocopherol (data not shown).

**Table 1** Protein kinase C (PKC) inhibitors or antioxidants relieved high free fatty acid (FFA)-induced inhibition of bradykinin-evoked nitric oxide (NO) production. Bovine aortic endothelial cells were cultured with indicated concentrations of FFAs for 5 or 10 days in the presence or absence of PKC inhibitors or antioxidants. Cells were stimulated by 1 µmol/l bradykinin for 90 min to determine NO production. Values are mean \(\pm\) S.E.M. of five independent experiments.

<table>
<thead>
<tr>
<th>Culture condition (control or FFAs)</th>
<th>NO production over 90 min (arbitrary unit)</th>
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<tbody>
<tr>
<td></td>
<td>No co-treatment</td>
</tr>
<tr>
<td>5 Days</td>
<td></td>
</tr>
<tr>
<td>1% BSA</td>
<td>7.45±0.22</td>
</tr>
<tr>
<td>0.5 mmol/l</td>
<td>5.67±0.96*</td>
</tr>
<tr>
<td>1.0 mmol/l</td>
<td>3.84±0.35†</td>
</tr>
<tr>
<td>10 Days</td>
<td></td>
</tr>
<tr>
<td>1% BSA</td>
<td>7.49±0.48</td>
</tr>
<tr>
<td>0.5 mmol/l</td>
<td>4.61±0.21†</td>
</tr>
<tr>
<td>1.0 mmol/l</td>
<td>3.07±0.31†</td>
</tr>
</tbody>
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*\(P<0.05\) and \(P<0.01\) vs individual control; †\(P<0.01\) vs corresponding fatty acid concentrations with no PKC inhibitor or antioxidant. These data were analyzed by ANOVA with Bonferroni post hoc correction.

Figure 5 Effects of FFAs on the morphology of BAECs. BAECs in coverslip chambers were cultured for 10 days (subcultured cells at the fifth day from seeding) under the following conditions: 1% BSA (A), 0.25 mmol/l (B), 0.5 mmol/l (C), and 1.0 mmol/l (D) fatty acids. Cell morphology was examined under differential interference contrast (DIC) microscopy. Each image is the representative of four independent experiments.
Discussion

In this study, we provide strong evidence that the PLC-activating receptor-Ca\(^{2+}\)-NO cascade pathway is impaired in ECs exposed to prolonged high concentrations of FFAs in a time- and dose-dependent manner. Furthermore, these alterations are probably in part due to a reduction of the affinity of receptors in ECs resulting in attenuated \([\text{Ca}^{2+}]_i\) responses. In BAEC, the dominant bradykinin receptor is B2 kinin subtype coupled to G-protein and PLC to activate eNOS to produce NO (Mombouli & Vanhoutte 1995, Wohlfart et al. 1997). Our results demonstrated the involvement of excessive activation of PKC and generation of oxidants in the FFA-induced impairments. We also observed similar findings when the effects of another PLC-activating receptor agonist (ATP) were studied.

Our data revealed that the impairments by high FFAs of both NO formation and \([\text{Ca}^{2+}]_i\) increment are specific to PLC-activating receptor agonists such as bradykinin and ATP, without impeding the actions of a Ca\(^{2+}\) ionophore or a specific inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase. These observations indicate that a step(s) in the signal transduction pathway between the stimulation of receptors and the activation of eNOS might be affected by FFAs. Our findings concur with the observations in the studies of diabetic animals and patients, in which the impaired endothelium-dependent vasodilation occurred only after the stimulation with receptor agonists such acetylcholine, but not following the application of NO donors or Ca\(^{2+}\) ionophores (Durante et al. 1988, Karasu & Altan 1993, Pieper 1999). Thus, a deficiency in NO, rather than a change in the sensitivity of vascular smooth muscles to NO, may make the major contribution to the impaired endothelium-dependent vasodilation in diabetes.

Our results suggest that an elevation of FFAs is not a regulator of eNOS expression in ECs, since FFA overload did not alter eNOS mass in cultured BAECs. These findings point that FFAs impaired the activity of eNOS rather than its expression, which is compatible with the observations by other investigators (Davda et al. 1995, Esenabhalu et al. 2003). eNOS activity is regulated by a complex combination of protein–protein interactions and the signal transduction cascade involving Ca\(^{2+}\) mobilization and phosphorylation events (Rubanyi & Vanhoutte 1988, Fleming & Busse 1999). A change in \([\text{Ca}^{2+}]_i\), represents a key determinant for eNOS redistribution and activity. eNOS is a Ca\(^{2+}\)–calmodulin-dependent enzyme and is transiently activated by an increase in \([\text{Ca}^{2+}]_i\), elicited by stimulation of diverse G-protein-coupled receptors.

Table 2  Reversal of high free fatty acids (FFA)-induced inhibition of bradykinin-evoked \([\text{Ca}^{2+}]_i\) rises by protein kinase C (PKC) inhibitors or antioxidants. Bovine aortic endothelial cells were cultured with various concentrations of FFAs for 5 or 10 days in the presence or absence of PKC inhibitors or antioxidants. Values are mean ± S.E.M. of five independent experiments.

<table>
<thead>
<tr>
<th>Culture condition (control or FFAs)</th>
<th>No co-treatment</th>
<th>Bisindolylmaleimide (10 μmol/l)</th>
<th>GÖ 6976 (20 μmol/l)</th>
<th>D-α-tocopherol (100 μmol/l)</th>
<th>N-acetyl-L-cysteine (10 μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% BSA</td>
<td>552 ± 58</td>
<td>522 ± 41</td>
<td>510 ± 28</td>
<td>564 ± 44</td>
<td>533 ± 55</td>
</tr>
<tr>
<td>0.5 mmol/l</td>
<td>399 ± 36*</td>
<td>498 ± 30†</td>
<td>486 ± 35‡</td>
<td>522 ± 51‡</td>
<td>504 ± 41‡</td>
</tr>
<tr>
<td>1.0 mmol/l</td>
<td>331 ± 29</td>
<td>464 ± 26§</td>
<td>450 ± 29§</td>
<td>499 ± 34§</td>
<td>472 ± 37§</td>
</tr>
<tr>
<td>10 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% BSA</td>
<td>547 ± 51</td>
<td>516 ± 47</td>
<td>504 ± 24</td>
<td>559 ± 32</td>
<td>526 ± 29</td>
</tr>
<tr>
<td>0.5 mmol/l</td>
<td>364 ± 33†</td>
<td>481 ± 34‡</td>
<td>450 ± 22‡</td>
<td>513 ± 36‡</td>
<td>499 ± 38‡</td>
</tr>
<tr>
<td>1.0 mmol/l</td>
<td>274 ± 37‡</td>
<td>452 ± 20‡</td>
<td>437 ± 39‡</td>
<td>500 ± 45‡</td>
<td>474 ± 42‡</td>
</tr>
</tbody>
</table>

*P<0.05 and †P<0.01 vs individual control; ‡P<0.01 vs corresponding fatty acid concentrations with no PKC inhibitor or antioxidant. These data were analyzed by ANOVA with Bonferroni post hoc correction.
in ECs. An increase in \([\text{Ca}^{2+}]_i\) can be a result of IP_3-mediated release of intracellularly stored \([\text{Ca}^{2+}]_i\) as well as \([\text{Ca}^{2+}]_i\) entry through nonvoltage-gated ion channels upon stimulation by receptor agonists (Rubanyi & Vanhoutte 1988, Freay et al. 1989). Our preceding data have demonstrated that NO productions in BAECs occurred in a \([\text{Ca}^{2+}]_i\) concentration-dependent manner, and stimulation of BAECs by various receptor agonists, such as bradykinin and ATP, increased eNOS enzymatic activity at least in part through an increase in \([\text{Ca}^{2+}]_i\). (Tang & Li 2004). In addition, our data revealed for the first time that FFA treatment was able to reduce the affinity of bradykinin receptor to its agonist, which might affect PLC activity and in turn reduce IP_3 production and \([\text{Ca}^{2+}]_i\) rise, as our previous work found that high glucose treatment affected the bradykinin receptor and also inhibited IP_3 production in BAECs (Tang & Li 2004).

There are a few studies reporting the short-term effects of FFAs on \([\text{Ca}^{2+}]_i\) in ECs (Kuroda et al. 2001, Esenabhalu et al. 2003, Tas et al. 2008) but no work has been done on the long-term effect. Acute incubation of ECs with FFAs reduced ATP- and histamine-induced \([\text{Ca}^{2+}]_i\) mobilization and \([\text{Ca}^{2+}]_i\) influx (Kuroda et al. 2001, Tas et al. 2008). Contradicting observations were obtained in another acute study (Esenabhalu et al. 2003), in which FFA-loaded (4 h) human umbilical vein ECs exhibited enhanced intracellular \([\text{Ca}^{2+}]_i\) release in response to stimulation by ATP, histamine, or thapsigargin, corresponding to an increase in intracellularly stored \([\text{Ca}^{2+}]_i\). Our work demonstrated that FFA overload for 5–10 days induced a time- and dose-dependent impairment of agonist-evoked \([\text{Ca}^{2+}]_i\) increments. However, such inhibitory effects could not be detected at a 2-day culture (cf. Supplementary Table 1). Most of the studies examining the temporal nature of the onset of endothelial dysfunction in experimental diabetes have shown a progressive worsening of dysfunction that appears to plateau at certain point of time. Dysfunction has been reported with an onset of 1 week of diabetes in rat intestinal arterioles (Lash & Bohlen 1991), 2 weeks in hindquarters (Kiff et al. 1991), 3 weeks in cremaster muscle arterioles (Alsip et al. 1996), and 4 weeks in aorta (Bucala et al. 1991). In addition, there is evidence for enhanced endothelial responses at an early stage (<2 days), which was followed by development of dysfunction at a late stage of diabetes (Pieper 1999, Johnstone & Caulfield 2001). Therefore, the findings that only long-term exposure to high FFAs reduced agonist-induced NO production and \([\text{Ca}^{2+}]_i\) increments in our study might be more relevant to the pathogenesis of dysfunctional endothelium occurring in diabetes.

How do high FFAs affect NO production in ECs in response to PLC-activating agonists? An elevation of FFAs would increase their uptake/transport and metabolism in BAECs. It is generally recognized that FFAs can enter ECs by passive diffusion, which is facilitated by the membrane fatty acid-binding protein (Goresky et al. 1994, Burczynski et al. 1995, van der Vusse et al. 2006, Imrie et al. 2006, Dichtl et al. 1999, Griffin et al. 1999, Inoguchi et al. 2000, Rask-Madsen & King 2007). It is possible that FFAs, similar to hyperglycemia, cause damage to ECs by overproduction of superoxide by the mitochondrial electron transport chain, leading to dysfunction in other metabolic pathways (Brownlee 2005, Du et al. 2006, Imrie et al. 2010). Infusion of FFAs

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**Table 3** Protein kinase C (PKC) inhibitors prevent free fatty acids (FFA)-induced reduction of bradykinin receptor affinity. Bovine aortic endothelial cells were cultured with indicated concentrations of FFAs for 10 days in the presence or absence of PKC inhibitors. Values are mean±S.E.M. of five independent experiments.

<table>
<thead>
<tr>
<th>Culture condition (control or FFAs)</th>
<th>No co-treatment</th>
<th>Bisindolylmaleimide (10 (\mu)mol/l)</th>
<th>(\alpha)-tocopherol (100 (\mu)mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% BSA</td>
<td>0.77±0.08</td>
<td>0.80±0.15</td>
<td>0.75±0.12</td>
</tr>
<tr>
<td>0.25 mmol/l</td>
<td>0.79±0.09</td>
<td>0.84±0.14</td>
<td>0.76±0.11</td>
</tr>
<tr>
<td>0.5 mmol/l</td>
<td>1.34±0.22*</td>
<td>0.88±0.13†</td>
<td>0.80±0.12†</td>
</tr>
<tr>
<td>1.0 mmol/l</td>
<td>1.46±0.16*</td>
<td>0.90±0.17†</td>
<td>0.81±0.21†</td>
</tr>
</tbody>
</table>

*\(P<0.01\) vs individual control; †\(P<0.01\) vs corresponding FFA concentrations with no PKC inhibitor. These data were analyzed by ANOVA with Bonferroni post hoc correction.
reduced endothelium-dependent vasodilation in animal models and in humans in vivo (Davda et al. 1995, Steinberg et al. 2000, Pleiner et al. 2002, Tripathy et al. 2003) and co-infusion of the antioxidant ascorbic acid improved endothelium-dependent vasodilation (Pleiner et al. 2002), indicating that oxidative stress might mediate the abnormality. Indeed, co-culture with antioxidants, in our study, was able to largely reverse the impairing effects of high FFAs on NO production and related signaling events in ECs.

FFAs might directly affect eNOS. It is known that phosphorylation of Ser 1179 in eNOS alters its activity, which is regulated by insulin through Akt. Literature has reported that FFAs may affect this scenario by inhibiting the insulin action, though FFA per se appeared not to affect Akt activity and eNOS phosphorylation at Ser 1179 (Lynn et al. 2004, Kim et al. 2005). Whether this scenario participates in high FFA-induced inhibition of PLC-activating agonist effects on $[\text{Ca}^{2+}]_\text{i}$, and eNOS remains to be investigated.

An elevation of FFA concentrations is also able to activate PKC (Griffin et al. 1999, Inoguchi et al. 2000, Rask-Madsen & King 2007, Ragheb et al. 2008), which may cause inhibition of endothelium-dependent vascular relaxation in vascular rings (Davda et al. 1994). Our results using PKC inhibitors suggested that excessive PKC activation is a candidate mechanism for the alterations of NO production, $[\text{Ca}^{2+}]_\text{i}$ elevation, and eNOS activity in response to agonists in ECs chronically exposed to FFA overload. Moreover, this proposed mechanism is supported by the previous work that observed that oleic acid could directly activate both typical PKC isoforms (by interacting with the regulatory domain at a site distinct from that for DAG) (el Touny et al. 1990) and the atypical PKC isoforms (Nakanishi & Exton 1992, Khan et al. 1993). Our studies also demonstrated that general PKC inhibitors were better than the isoform-specific PKC inhibitor for reversing FFA-induced impairments, suggesting the possible involvement of both $\text{Ca}^{2+}$-dependent and $\text{Ca}^{2+}$-independent PKC isoforms. PKC could inhibit PLC-implicated signaling pathway by affecting G-proteins (Brock & Capasso 1988, Ryu et al. 1990), while ROS might directly affect signaling partners and cause the membrane lipid peroxidation (Brownlee 2001, Stentz & Kitabchi 2006), leading to aberrant coupling of receptor to G-proteins to PLC.

The morphological observations in this study revealed that pathophysiological concentrations of FFAs might disturb the integrity of EC membrane, which, if it occurs in vivo, may cause adverse effects on vascular fluidity and the interaction of ECs with immune cells and platelets. Interestingly, this scenario seemed to indicate an involvement of PKC activation and oxidative stress since $\alpha$-tocopherol could reverse the effect.

### Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1550/JME-11-0082.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

### Funding

This work was supported by a grant from the National Medical Research Council of Singapore (NMRC/1218/2009 to G.L).

### Author contribution statement

G.L. initiated the study, organized the experiments, evaluated the data, and wrote the manuscript. Y.T. designed and performed experiments, analyzed the data, and wrote the manuscript.

### Acknowledgements

We are very grateful to Dr Stewart Metz for the editorial help. We thank C.I. Teo and R Luo for skillful technical assistance. Y.T. was a recipient of research scholarship from the National University of Singapore.

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Received in final form 4 October 2011
Accepted 12 October 2011
Made available online as an Accepted Preprint 12 October 2011


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