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Free Fatty Acid Overload Attenuates Ca^{2+} Signaling and NO Production in Endothelial Cells

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ABSTRACT

Hyperlipidemia represents a major risk factor for development of vascular dysfunction and atherosclerosis. Although the unfortunate role of low-density lipoprotein has been clearly demonstrated, the mechanistic pathways through which triglyceride-derived free fatty acids (FFAs) contribute to vascular disorders are not completely understood. Thus, the present study was designed to elucidate the effects of FFAs on cultured endothelial cells. The Ca^{2+} signaling, endothelial nitric oxide synthase (eNOS) activity, and production of superoxide anions ($\cdot O_2^-$) were monitored in cells treated with bovine serum albumin-conjugated FFA. FFA-loaded cells showed enhanced intracellular Ca^{2+} release in response to ATP, histamine, or the SERCA inhibitor thapsigargin. This effect corresponded to an overall increase in intracellularly stored Ca^{2+}. In contrast, autacoid-triggered elevation of cytosolic free Ca^{2+} concentration was blunted in FFA-loaded cells due to inhibition of capacitative Ca^{2+} entry. In agreement with the reduced Ca^{2+} signaling, the Ca^{2+}-dependent activity of eNOS was reduced under basal conditions and if cells were stimulated with ATP, histamine, or thapsigargin. The attenuated eNOS activity was associated with $\cdot O_2^-$ release in FFA-loaded cells. These data indicate that FFAs significantly affect endothelial Ca^{2+} signaling, eNOS activity, and $\cdot O_2^-$release and, thus, might contribute to vascular dysfunction in atherogenesis. *Antioxid. Redox Signal.* 5, 147–153.

INTRODUCTION

DESPITE THE VARIOUS BIOLOGICAL FUNCTIONS OF FREE FATTY ACIDS (FFAs), disturbances in FFA metabolisms have been implicated in several human vascular diseases. Particularly, the concept that abnormalities in fatty acid metabolism may be the primary metabolic disturbance in non–insulin-dependent diabetes mellitus and obesity has gained momentum over the last decade (5). Vascular disorders, such as atherosclerosis, have been known to account for more premature deaths and long-term disability than any other disorder in industrialized society. The pathologic state of atherosclerosis becomes macroscopically visible in so-called atherosclerotic plaques that represent initially an accumulation of cholesterol on the inside of the arterial wall. To uncover the molecular mechanisms that contribute to early "fatty streak formation," macrophage invasion and smooth muscle proliferation, which promote plaque formation, still remain a challenge to vascular science research. Furthermore, whereas the atherosclerotic risk of low-density lipoprotein (3), inflammation (7), or bacterial infection (21) has been clearly demonstrated, the role of FFAs is still under debate. Notably, in a transgenic mouse model with tissue-specific expression of human lipoprotein lipase in the heart and the circulatory system (19), large accumulation of FFAs was associated with severe blood vessel dysfunction (6). In this model, endothelial-dependent relaxation constituted the primary phenomenon, whereas smooth muscle reactivity was only slightly affected. These data are in agreement with the common hypothesis that dysfunction of endothelial cells is involved already in the initial phase of atherogenesis or even represents a primary target of risk factors that are associated with atherosclerosis.

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Therefore, a study was designed to assess the effect of FFA overload on autacoid-triggered Ca^{2+} signaling, endothelial nitric oxide synthase (eNOS) activation, and free radical production in the human umbilical vein endothelial cell line EA.hy926.

MATERIALS AND METHODS

Materials

Cell culture chemicals were obtained from Life Technologies (Vienna, Austria). Fetal calf serum (premium quality) and donor horse serum were from PPA Laboratories (Linz, Austria). Petri dishes and cell culture plastic were from Iwaki (Bertoni, Vienna, Austria). Fura-2/AMwas purchased from Molecular Probes (Leiden, Netherlands). Buffer salts were from Merck (Vienna, Austria). All other materials were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

The human umbilical vein endothelial cell line EA.hy926 was a kind gift from Dr. Cora-Jean S. Edgell (Pathology Division, University of North Carolina, Chapel Hill, NC, U.S.A.). Endothelial cells of passages higher than 43 were used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% HAT (5 m*M* hypoxanthine, 20 μ*M* aminopterin, and 0.8 m*M* thymidine).

Preparation of sodium oleate–albumin complex solution (SOACS)

In an attempt to produce a high FFA loading in cells, endothelial cells (EA.hy926) were preincubated in SOACS. This solution is prepared by modification of the method of Van Harken *et al.* (31). Oleic acid (22.5 mg) was transfered to a 20-ml beaker containing 0.5 ml of ethanol, and 25 μl of 5 *M* NaOH was added followed by thorough mixing. The ethanol was removed by evaporation under nitrogen, and 2.5 ml of 150 m*M* NaCl solution was added. The clear solution was heated for 3–5 min at 60°C. While the fatty acid solution was still warm, 3.1 ml of ice-cold 24% (wt/vol) bovine albumin in 150 m*M* NaCl was added rapidly and stirred for 10 min. The final volume was adjusted to 6.25 ml with 24% (wt/vol) bovine albumin in 150 m*M* NaCl, and the SOACS was kept frozen at −20°C until use. The 24% (wt/vol) bovine albumin solution was prepared by adding 3 g of bovine serum albumin (Sigma Chemicals, Vienna, Austria) to 8.8 ml of 150 m*M* NaCl. The albumin was added in six aliquots (0.5 g per aliquot) over a 5-h period while the mixture was being stirred at room temperature. The solution was adjusted to a final pH of 7.4 with 5 *M* NaOH, and final volume was adjusted to 12.5 ml with 150 m*M* NaCl. To perform a concentration–response correlation analysis, SOACS was diluted with equally concentrated bovine serum solution. The solution was kept at −20°C until use. In general, cells were preincubated with SOACS for 4 h in culture medium. Control cells were treated with the bovine serum albumin at the same concentration (*i.e.,* 2.4%).

Ca^{2+} measurement

Intracellular Ca^{2+} concentration was determined using Ca^{2+}-dye fluorescence techniques for fura-2 as previously described (13). Cultured cells were harvested by 3 min of trypsin, centrifuged, resuspended in DMEM containing 2 μ*M* fura-2/am, and kept at room temperature. After 45 min at room temperature, cells were centrifuged and the DMEM changed and allowed another 10 min at room temperature. One milliliter of the cell suspension was transferred into a test tube, centrifuged, and resuspended in 2 ml of HEPES-buffered solution containing the following (in m*M*): 2.5 $CaCl_2$, 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES acid, 10^{-5} EGTA (pH adjusted to 7.4). Intracellular Ca^{2+} was monitored in suspended cells in a thermostatically controlled fluorescence spectrometer (Hitachi F-2000). Changes in Ca^{2+} concentration were monitored by measuring the ratio of fluorescence at 340 and 380 nm excitation (F_{340}/F_{380}) and 510 nm emission. For calculation of the nanomolar free Ca^{2+} concentration from the ratio (F_{340}/F_{380}) units obtained, an *in situ* calibration curve was used as previously described (9).

Measurement of eNOS activity

Activity of eNOS (in the FFA and control groups) was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, similar to the procedure described previously (15, 23). Endothelial cells of the two groups were cultured in six-well plastic dishes. At confluence, cultured medium was removed, and cells were washed twice with phosphate buffer solution containing the following (in m*M*): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4 (pH adjusted to 7.4). The experiment was commenced by the addition of 900 μl of HEPES buffer plus 2.5 m*M* Ca^{2+}, containing 2×10^6 d.p.m. L-[^{3}H]arginine and 100 μl of either buffer (*i.e.,* control) or agonists, histamine (10 μ*M* final), thapsigargin (1 μ*M* final), and ATP (10 μ*M*). In parallel series, experiments were repeated in the presence of 300 μ*M* L-N^G-nitro-arginine and performed for each condition. After a 15-min incubation period, buffer was discarded, cells were washed three times with chilled Ca^{2+}-free HEPES buffer, and lysed with 1 ml of 0.01 *M* HCl. After 1 h at 4°C, the total incorporated radioactivity was measured in a 100-μl aliquot. The remaining 0.9 ml of HCl was buffered with 100 μl of 200 m*M* sodium acetate buffer (pH 13.0) containing 10 m*M* L-citrulline. The amino acids L-[^{3}H]arginine and L-[^{3}H]citrulline were separated using column chromatography as described recently (15, 23). Activity of eNOS was calculated by the L-N^G-nitro-arginine-sensitive percentage of the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline.

Superoxide anion ($\cdot O_2^-$) measurements

As described previously (8), confluent EA.hy926 cells preincubated with 150 μl of SOACS and control cells were used in these experiments. Cells were harvested with trypsin (2 min), centrifuged, and resuspended in 990 μl of phosphate buffer solution and 10 μl of ferricytochrome C solution (1 m*M*). After the respective incubation time, cells were centrifuged and the supernatant was transferred into a cuvette for measurements. Immediately after the cells were mixed with the ferricytochrome C solution, a first reading was taken fol-

lowed by an incubation period of 1 h. Thereafter, superoxide dismutase (SOD; 500 U/ml) was added, and an intimate reading following by an incubation time of 15 min prior to the last readings was performed.The $\cdot O_2^-$-release from the FFA-loaded endothelial cells was monitored in the spectrophotometer at 550 nm emission. Differences in extinction in the absence and presence of SOD [$E_{O2}- = E_{-SOD} - 4 \times (E_{+SOD})$] represent $\cdot O_2^-$-related reduction of ferricytochrome C and can be calculated using the molar extinction coefficient of the reduced form of ferricytochrome C (ε = 21.000) (29).

Statistics

Data are expressed as means ± SE. Statistical analysis was performed using paired samples *t* test with Bonferroni correction for multiple comparisons. Data are considered significant if $p < 0.05$.

RESULTS

In control cells, addition of 1 μ*M* histamine evoked a fast and transient Ca^{2+} elevation followed by a long-lasting and stable plateau phase (Fig.1A). In contrast, in SOACS-treated cells, the initial transient Ca^{2+} elevation in response to 1 μ*M* histamine was reduced by ~25% and no plateau phase occurred (Fig. 1A). In order to differentiate between intracellular Ca^{2+} release and Ca^{2+} influx, endothelial cells were stimulated in the nominal absence of extracellular Ca^{2+} with 1 μ*M* histamine (*i.e.,* intracellular Ca^{2+} release), followed by a readdition of 2.5 m*M* extracellular Ca^{2+} (*i.e.,* Ca^{2+} entry). Notably, histamine-induced intracellular Ca^{2+} release was slightly increased in SOACS-treated cells (Fig. 1B), whereas Ca^{2+} entry was prevented in FFA-loaded cells (Fig. 1C). Furthermore, a concentration-response curve for histamine was conducted in control cells and in SOACS-treated cells (Fig. 2A). In general, FFA loading reduced the maximal effect by ~25%, whereas the EC_{50} remained unchanged [control: 0.42 (0.11–1.57) μ*M*, *n* = 12; SOACS-treated: 0.70 (0.23–2.11) μ*M*, *n* = 11].

In agreement with our data obtained with histamine, treatment of endothelial cells with SOACS reduced ATP-evoked Ca^{2+} signaling by ~20%, whereas the EC_{50} for ATP was unaffected by the FFA-loading procedure [control: 2.22 (1.62–3.04) μ*M*, *n* = 17–22; SOACS-treated: 2.41 (1.73–3.35) μ*M*, *n* = 17–21]. Furthermore a concentration-effect correlation for SOACS on endothelial Ca^{2+} signaling evoked by 1 μ*M* histamine was performed (Fig. 3).

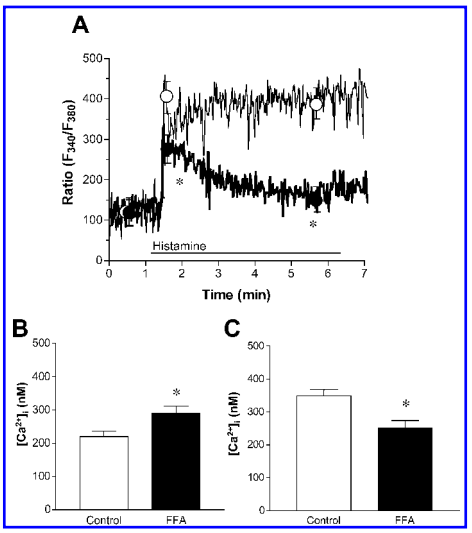

FIG. 1. Effect of SOACS treatment on endothelial Ca^{2+} signaling in response to histamine. Cultured endothelial cells were treated for 4 h in culture medium containing 2.4% bovine serum albumin without and with oleic acid (360 μg/ml oleic acid; FFA). Final oleic acid concentration was 360 μg/ml. (**A**) Original tracing of the Ca^{2+} signaling initiated by 1 μ*M* histamine in Ca^{2+}-containing solution in control (thin line, μ; *n* = 8) and SOACS (thick line, λ; *n* = 8) treated cells. (**B**) Intracellular Ca^{2+} release in response to 1 μ*M* histamine in control (*n* = 18) and FFA-loaded cells (*n* = 16). (**C**) Histamine-induced Ca^{2+} entry visualized by addition of 2.5 m*M* extracellular Ca^{2+} to prestimulated cells (control, *n* = 18; FFA, *n* = 16). **p* < 0.05 vs. control.

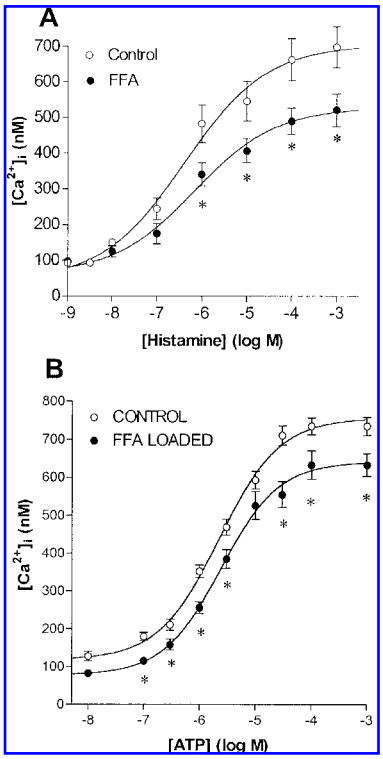

FIG. 2. Effect of FFA loading on sensitivity to histamine and ATP to evoke Ca^{2+} signaling. Endothelial cells were incubated for 4 h with bovine serum albumin (control, μ) or SOACS (360 μg/ml oleic acid; FFA, λ). Concentration-response curves to histamine (**A**; control, *n* = 12; FFA, *n* = 11) and ATP (**B**; control, *n* = 17–22; FFA, *n* = 17–21) were performed in 2.5 m*M* Ca^{2+} containing buffer. **p* < 0.05 vs. control.

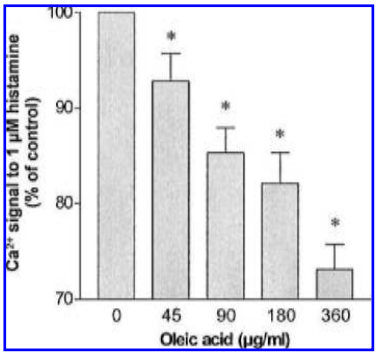

FIG. 3. Concentration-response correlation for SOACS on histamine-induced signaling. Cultured endothelial cells were preincubated for 4 h with SOACS containing the final oleic acid concentrations indicated. Intracellular Ca^{2+} signaling in response to 1 μ*M* histamine was monitored and expressed as % of that measured in cells treated with bovine serum albumin alone (n = 17–20). *$p < 0.05$ vs. control.

In order to investigate whether the inhibitory effect of the SOACS treatment on endothelial Ca^{2+} signaling was due to an inhibitory effect on receptor–effector coupling, the effect of the smooth endoplasmic reticulum Ca^{2+} antiporter (SERCA) inhibitor thapsigargin was tested. As shown in Fig. 4A, the effect of 1 μ*M* thapsigargin to elevate cytosolic free Ca^{2+} concentration in the presence of extracellular Ca^{2+} was strongly reduced in SOACS-treated cells. A further evaluation in a Ca^{2+}-free environment revealed an increased intracellular Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores in FFA-loaded cells (Fig. 4B), whereas thapsigargin-initiated capacitative Ca^{2+} entry (CCE) was reduced by ~73% (Fig. 4C).

Thus, these data with thapsigargin supported our findings that SOACS loading results in an overall increase in releasable Ca^{2+} in intracellular Ca^{2+} pools (presumably the endoplasmic reticulum), whereas CCE is strongly inhibited. To evaluate the amount of overall releasable Ca^{2+} stored in intracellular Ca^{2+} pools, endothelial cells were challenged by the Ca^{2+} ionophore ionomycin. In control cells, 100 n*M* ionomycin increased $[Ca^{2+}]_i$ in the nominal absence of extracellular Ca^{2+} up to 571 ± 11 n*M* (n = 12). In SOACS-treated cells, the effect of 100 n*M* ionomycin to elevate $[Ca^{2+}]_i$ in nominal Ca^{2+}-free solution was enhanced (643 ± 7 n*M*, n = 14; $p <$ 0.05 versus control cells). Remarkably, CCE visualized by the addition of extracellular Ca^{2+} to ionomycin-treated cells (100 n*M*) did not differ within the control and SOACS-treated cells (data not shown).

To evaluate further the effect of SOACS loading on endothelial vascular function, eNOS activity measurements were performed. Basal eNOS activity was reduced by 70% in FFA-loaded cells (Fig. 5A). Hence, the stimulatory effects of

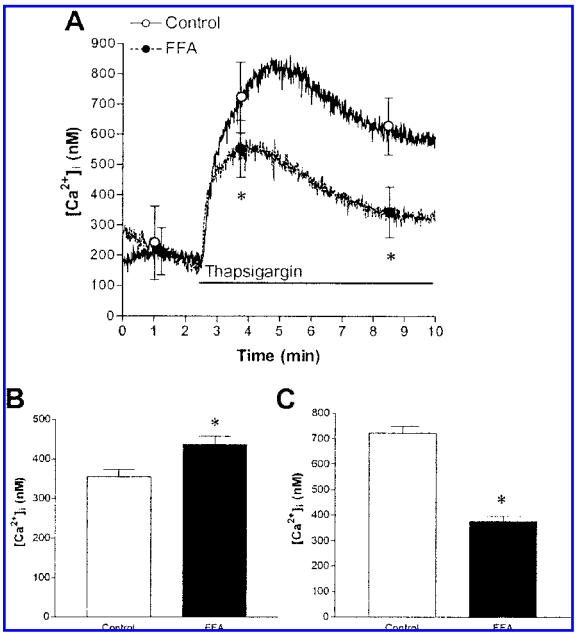

FIG. 4. Effect of SOACS treatment on endothelial Ca^{2+} signaling in response to thapsigargin. After a 4-h incubation with or without SOAC (360 μg/ml oleic acid), cells were loaded with fura-2/AM and changes in intracellular free Ca^{2+} concentration due to 1 μ*M* thapsigargin in Ca^{2+}-containing buffer (**A**; control, n = 13; FFA, n = 14) and in Ca^{2+}-free solution (**B**; control, n = 15; FFA, n = 12) were monitored. (**C**) Thapsigargin-induced Ca^{2+} entry visualized by addition of 2.5 m*M* extracellular Ca^{2+} to prestimulated cells (control, n = 18; FFA, n = 16). *$p < 0.05$ vs. control.

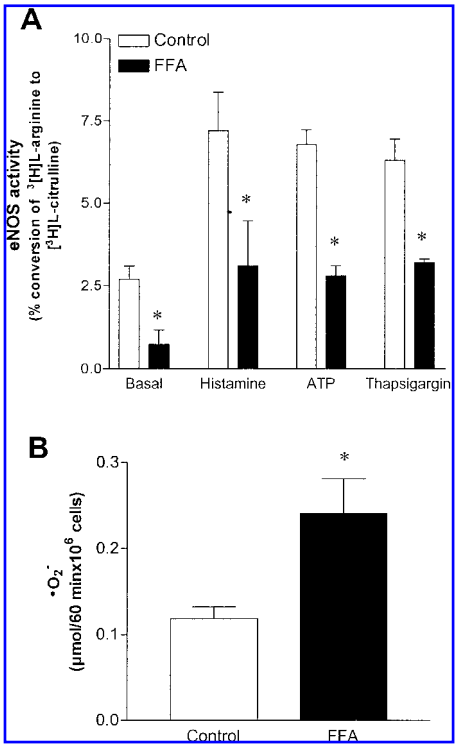

FIG. 5. Alterations of eNOS activity and $\cdot O_2^-$ release in endothelial cells due to SOACS treatment. Activity of eNOS (**A**; control, n = 4; FFA, n = 4) and the release of $\cdot O_2^-$ (**B**; control, n = 6; FFA, n = 6) were measured in cultured endothelial cells pretreated for 4 h with 2.4% bovine serum albumin or SOACS (360 μg/ml oleic acid). *$p < 0.05$ vs. control.

histamine (10 μ*M*), ATP (100 μ*M*), and thapsigargin (1 μ*M*) were diminished in SOACS-treated cells and did not exceed basal eNOS activity in the control cells (Fig. 5A). The reduced eNOS activity in SOACS-loaded endothelial cells was further associated with an increased release of $\cdot O_2^-$ by ~102% (Fig. 5B).

DISCUSSION

Hyperlipidemia as a risk factor for vascular dysfunction has been widely reported. A pathologic state characterized by accumulation of fatty materials on the inside of the arterial wall to form plaque is evident in atherosclerosis (33). In addition, the concept that abnormalities in fatty acid metabolism may be the primary metabolic disturbance in non–insulin-dependent diabetes and obesity has gained momentum over the last decade (5). Although several literature reports have implicated FFA overload in many human vascular diseases, the mechanistic pathways involved in the development of vascular dysfunction have remained unresolved. Based on our previous report that revealed vascular dysfunction in an animal model overexpressing human lipoprotein lipase in the mouse vascular bed, which was accompanied by increased FFA content in the vasculature (6), in the present study we have tested the impact of FFA loading on various cell functions of cultured endothelial cells. We have elucidated the effects of FFA overload of the endothelial cells on autacoid-evoked Ca^{2+} signaling and eNOS activity and assessed the release of $\cdot O_2^-$ in response to FFA overload.

Autacoid-induced Ca^{2+} signaling in endothelial cells was significantly reduced in FFA-loaded cells. Interestingly, the reduction was not due to a receptor-desensitizing phenomenon as the EC_{50} for either histamine or ATP did not differ between control and FFA-loaded cells. On the other side, the reduction of Ca^{2+} increases evoked by one given agonist concentration was attenuated in the FFA-loaded cells over the whole concentration range tested. Despite this clear reduction in Ca^{2+} elevation upon autacoids in the presence of extracellular Ca^{2+}, a more detailed analysis of the correlation of FFA loading and endothelial Ca^{2+} signaling revealed an increased intracellular Ca^{2+} release upon stimulation with histamine, whereas CCE, which was visualized by the addition of extracellular Ca^{2+} to cells that were prestimulated in Ca^{2+}-free buffer, was strongly reduced. We have previously shown that pretreatment of endothelial cells with elevated D-glucose resulted in elevated inositol 1,4,5-trisphosphate formation, which, in turn, resulted in an enhanced intracellular Ca^{2+} release in response to agonists (32). However, intracellular Ca^{2+} release induced by thapsigargin, which depletes endoplasmic reticulum independently of inositol 1,4,5-trisphosphate by inhibition of SERCA, was elevated in FFA-loaded cells. Furthermore, ionomycin-triggered intracellular Ca^{2+} release was also found to be enhanced in SOACS-treated cells. These data point to an increased Ca^{2+} content of the endoplasmic reticulum and/or increased endoplasmic reticulum lumen in cells loaded with FFA.

Despite the increase in intracellular stored Ca^{2+} in FFA-loaded cells, histamine-activated CCE, which was visualized by the addition of extracellular Ca^{2+} to prestimulated cells, was reduced by FFA loading. In agreement with these results, the receptor-independent CCE evoked by thapsigargin was strongly diminished in SOACS-treated endothelial cells. These data demonstrate that an overload of endothelial cells results in reduction of CCE activity independently of intracellular Ca^{2+} release processes. The mechanisms of CCE activation in endothelial cells remain a mystery, although various hypotheses have been suggested (2). Among them, protein–protein coupling between the putative Ca^{2+} channels (35) and the inositol 1,4,5-trisphosphate receptor (2), channel trafficking (28), and several diffusible factors have been proposed to be responsible for transmitting a signal for channel activation if the endoplasmic reticulum becomes depleted (24, 25). As diffusible factors that were proposed to activate CCE, a small nonprotein factor processing a phosphate group (26) that is degradated by Ca^{2+} and phosphatases (27) and arachidonic acid derivates from the epoxygenase (1, 14, 16) or lipoxygenase (12) pathway have been suggested. Common to all proposed diffusible factors is that their release/production/activation occurs upon depletion of the endoplasmic reticulum. As it became obvious that FFA-loading did not affect intracellular Ca^{2+} release and depletion of the endoplasmic reticulum by the inositol 1,4,5-trisphosphate-activating autacoids histamine and ATP, as well as the SERCA inhibitor thapsigargin (30), it seems unlikely that the reduced CCE in SOACS-loaded cells is due to alterations in Ca^{2+} release processes. Thus, the reduced Ca^{2+} entry might be due to an alteration in CCE activation or in the activity of the channels that are responsible for the CCE. Although the latter seems reasonable as changes in FFA composition of the membrane due to SOACS treatment might affect channel function, our findings that CCE in response to 100 n*M* ionomycin did not differ between the control and the SOACS-treated cells suggest that mechanisms of CCE activation and the channel activity remained unchanged in FFA-loaded cells. If neither the activation mechanisms nor the activity of CCE is altered, how does SOACS treatment affect endothelial CCE? Recently, mitochondrial Ca^{2+} signaling got attention for CCE activation as it became obvious that mitochondrial Ca^{2+} sequestration crucially facilitates CCE by buffering subplasmalemmal Ca^{2+} concentration to avoid Ca^{2+}-mediated inactivation of the CCE channels (10, 11, 17, 18). Notably, as a consequence of its property to act as a Ca^{2+} ionophore, ionomycin-induced CCE was found to be independent of this mitochondrial Ca^{2+} buffering function. As mitochondrial respiratory functions and proliferation are controlled by FFAs (34), it is tempting to speculate that SOACS loading affects mitochondrial Ca^{2+} buffering function and, thus, prevents CCE. Furthermore, saturated but not unsaturated FFAs have been described to contribute to a Ca^{2+}-activated ion pore in the mitochondria that might be responsible for the rapid Ca^{2+} sequestration of the mitochondria (22). An overload with oleic acid by SOACS treatments might affect mitochondrial FFA content and, thus, the formation of Ca^{2+}-activated ion pores in the mitochondrial membrane, thus preventing mitochondrial Ca^{2+} buffering and, in turn, CCE activity. Further studies are necessary to prove the impact of FFA overload on mitochondrial Ca^{2+} function.

The stimulation of eNOS activation has been found to be essential due to an activation of Ca^{2+} influx (20). In line with these reports, SOACS treatment that blunted CCE further di-

minished eNOS activation in response to histamine, ATP, and thapsigargin. Interestingly, basal eNOS activity was found to be reduced in SOACS-treated cells also. Whether this effect is due to the attenuation of basal Ca^{2+} influx in FFA-loaded cells, the lack of supplementary cofactors, or alterations in eNOS distribution is unclear and the subject of further studies. Nevertheless, these findings are in agreement with our recent report that increased FFA content of blood vessels reduce nitric oxide production (6). In line with this previous work, we also have found an increased release of $\cdot O_2^-$. An increased $\cdot O_2^-$ release in the vasculature has been proven to scavenge nitric oxide to be active in the smooth muscle cells (4), thus promoting vascular dysfunction and atherogenesis.

The reported alterations of endothelial function by FFA overload further indicate that FFA contribute to vascular complications. Although the molecular mechanisms of action need further investigation, the consequences on vessel reactivity clearly point to a reduction of the biosynthesis and the bioactivity of nitric oxide.

ABBREVIATIONS

CCE, capacitative Ca^{2+} entry; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; FFA, free fatty acid; $\cdot O_2^-$, superoxide anion; SERCA, smooth endoplasmic reticulum Ca^{2+} antiporter; SOACS, sodium oleate–albumin complex solution; SOD, superoxide dismutase.

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