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On the validity of fluorimetric intracellular calcium detection: Impact of lipid components

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ABSTRACT

We investigated the effects of different lipids on the activity of the angiotensin II type 1 receptor (AT1R). As calcium plays a key role in the signaling of the AT1R, we used the calcium-sensitive fluorescence indicators fura-2 to detect intracellular calcium release upon stimulation with the agonist angiotensin II. At first sight, cells preincubated with Very low-density lipoprotein (VLDL) showed a reduced calcium release triggered by angiontensin II compared to untreated control. However, on closer examination, this result seemed to be an artifact. Incubation with VLDL reduced also the amount of intracellular fura-2, as measured by fluorescence in the isosbestic point. Additionally, the maximal obtainable ratio, obtained after complete saturation with calcium ions, was reduced in cells preincubated with VLDL. These findings rendered our initial results questionable. We report the results of our work and our suggestions regarding the experimental setup to contribute to the understanding of the interpretation of fura-2 measurements and to avoid erroneous conclusions.

1. Introduction

Calcium plays a key role in numerous cellular signaling processes, e.g. as a second messenger in G-protein coupled receptor (GPCR)-signaling [1] in gene expression [2], apoptotic or necrotic cell death [3]. Therefore, calcium detection techniques are of major interest. Intracellular calcium can be measured by fluorescent dyes [4]. There are two types of fluorescent dyes: non-ratiometric dyes and ratiometric dyes.

In non-ratiometric dyes (e.g. fluo-4), the intensity of the fluorescence changes upon calcium binding, and the fluorescence spectrum does not shift. With these dyes, intensity changes that result from factors unrelated to changes in Ca^{2+}, like uneven dye concentration, dye leakage or cell number, may confound the interpretation of the data [4].

In contrast, ratiometric dyes (e.g. fura-2) present a fluorescence spectrum that shifts its peak upon calcium binding. For evaluation, a ratio of two wavelengths is used, e.g. in the case of fura-2, the fluorescence measured at 340 nm is divided by the fluorescence measured at 380 nm. Confounders like uneven dye concentration, dye leakage, cell number etc. cancel themselves out as they the influence the fluorescence at both wavelengths similarly [4].

We aimed to investigate the effects of very-low density lipoprotein (VLDL) and two defined fatty acids, the saturated stearic acid and the unsaturated oleic acid, on the activity of the angiotensin II type I receptor (AT1R), reported by intracellular calcium release as detected by the indicator fura-2.

The AT1R is a GPCR, which signals among others through the second messenger calcium and further downstream signals [5]. The AT1R mediates the effects of angiotensin II, a critical regulator of blood volume, cardiovascular homeostasis and blood pressure, and plays a pivotal role in the development of cardiovascular diseases like hypertension and atherosclerosis [5,6]. Lipid metabolism is closely related to endothelial dysfunction and subsequent

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cardiovascular disease [7]. For plasma lipoproteins like low-density lipoprotein (LDL) an impact on the AT1R-functionality in vitro and in vivo has already been proposed [8–10].

During our analyses, we encountered methodological difficulties in measuring and interpreting intracellular calcium release in cell pre-incubated with VLDL in our experimental setup. This aligns with several reports that the detection of calcium with fura-2 is not without pitfalls. For example, the presence of amlodipine or resveratrol may lead to misinterpretations of calcium determination with fura-2 due to interferences of the tested compounds with a fura-2 wavelength because of the high autofluorescence of these compounds [11–13].

High amounts of lipids outside the adipose tissue may have detrimental effects in vivo and in vitro [14]. This effect of lipotoxicity seems to play a role in our case. Here, we report the results of our work to contribute to the understanding of the interpretation of fura-2 measurements in another experimental setting and to avoid erroneous conclusions from calcium measurements.

2. Methods

2.1. Cell culture

CHO-K1 transfected with AT1R (GenBank NM_000685.4, Perkin Elmer Waltham, USA) were grown in Ham's-F12 Medium (Biochrom, Berlin, Germany), supplemented with 10% Fetal Bovine Serum Superior (Biochrom, Berlin, Germany), 0.4 mg/mL Geneticin (Gibco LifeTechnologies, Darmstadt, Germany) and 0.25 mg/mL Zeocin (Invivogen, San Diego, USA) according to the manufacturer's instructions and subcultured at 90% confluence. Cells up to passage 30 were used.

2.2. Preparation of very-low-density lipoprotein

Venous EDTA-plasma samples were collected from healthy volunteers after an overnight fast. Blood sampling was approved by the local ethics committee and subjects gave written informed consent (local ethics committee number 140/13 and 443/20). The plasma was subjected to preparative sequential density ultracentrifugation with a target density less than 1.006 kg/L for very low-density lipoprotein (VLDL) [15]. The resulting fraction of VLDL was desalted through PD-10 exchange columns (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions: After equilibration of the columns with 25 mL of phenol-red-free RPMI-Medium (Biochrom. Berlin, Germany), amounts of 2.5 mL were loaded to the columns and resulting eluent was discarded, the fractions were then eluted with additional 3 mL of RPMI. 50 μM BHT (SigmaAldrich, Steinheim, Germany) was added as antioxidant. The respective eluted fractions were collected and sterile-filtered through a 0.22 μM PVDF-Membrane filter (MerckMillipore, Darmstadt, Germany) and measured for ApoB content on an automated platform (Olympus AU 640, BeckmanCoulter Krefeld, Germany) with commercially available test kits (DiaSys Greiner, Flacht, Germany). The amount of VLDL is given as ApoB in μg/mL.

2.3. Preparation of fatty acid solutions

Stock solutions with a final concentration of 90 mM of the fatty acids stearic acid (C 18:0) and oleic acid (C 18:2) (SigmaAldrich, Steinheim, Germany) were prepared in ethanol (SigmaAldrich, Steinheim, Germany). The stock solutions were diluted to the required concentration with 40 mg/mL bovine serum albumin (BSA) in Ham's F12 medium.

2.4. Measurement of intracellular calcium

20.000 cells per well were seeded in black, clear bottom 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and grown overnight, changed to serum-free medium the next day and incubated 24 h later for further 24 h with VLDL, fatty acid solutions or control solutions. Cells were then washed twice with 100 μL PBS, and loaded with 5 μM FURA-2-AM in serum-free medium containing 0,1% fatty acid free BSA for 30 min at 37 °C. Cells were then washed twice with PBS (Biochrom, Berlin, Germany).

100 μL of assay buffer consisting of HBSS (Gibco, LifeTechnologies, Darmstad, Germany), 20 mM HEPES (SigmaAldrich, Steinheim, Germany), 2.5 mM Probenecid (SigmaAldrich, Steinheim, Germany) was added. Cells rested for another 30 min in the dark at room temperature to allow for complete deesterification. Angiotensin II solution consisted of angiotensin II (SigmaAldrich, Steinheim, Germany) at a final concentration of 2 nM in HBSS with 20 mM HEPES. Ionomycin solution consisted of ionomycin (SigmaAldrich, Steinheim, Germany) and calcium at a final concentration of 10 μM and 10 mM respectively in HBSS with 20 mM HEPES.

Measurements were performed in a VarioskanFlash multiplate reader (Thermo Fischer Scientific, Karlsruhe, Germany) at alternating excitation wavelengths of 340, 360 and 380 nm and a fixed emission wavelength of 510 nM. The instrument injected stimulating solutions and recorded the fluorescence before and after the injection. Measurements are converted to the fluorescence ratio (R), dividing the arbitrary fluorescence units (AU) at 340 nm (Ca^{2+} bound fura-2) by the respective AU obtained at 380 nm (unbound fura-2), R= *fluorescence at 340/fluorescence at* 380 nm. All samples and controls were measured at least in in duplicates.

2.5. Measurement of LDH-release

Cytotoxic effects of the compounds were measured by release of lactatedehydrogenase (LDH) into the supernatant (cytotoxicity detection kit (LDH), Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. 20.000 cells per well were seeded in clear 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and grown overnight. The next day the medium was changed to serum-free medium for 24 h. Cells were then loaded for 24 h with different concentrations of VLDL, fatty acids negative control (medium only) or positive control (Triton 1%). 100 μL of cell culture supernatants were transferred to clear 96-well plates and 100 μL of reaction mixture (consisting of catalyst and INT dye solution) was added. The plate was measured within 30 min at 490 nm with a reference wavelength of 690 nm. Results are given as A_{490}-A_{690} in relation to the LDH-releasing positive control 1% TritonX-100, which was set as 100%. Measurements were performed in duplicates.

2.6. Measurement of cell viability

Cell viability was measured with the MTT assay (Cell proliferation kit I (MTT), Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. 20.000 cells per well were seeded in clear 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and grown overnight. The next day the medium was changed to serum-free medium for 24 h. Cells were then loaded for 24 h with different concentrations of VLDL, fatty acids, negative control (medium only) or positive control (Actinomycin D 10 μM). Cells were washed two times with 100 μL PBS Dulbecco (Biochrom, Berlin, Germany). 100 μL of assay buffer consisting of HBSS (Gibco, LifeTechnologies, Darmstad, Germany), 20 mM HEPES (SigmaAldrich, Steinheim, Germany), 2.5 mM Probenecid (SigmaAldrich, Steinheim, Germany) were pipetted in each well. 10 μL of MTT-

labeling reagent was added and incubated for 4 h in a humidified atmosphere at 37 °C and 5% CO_2. 100 μL of solubilization solution was added and incubated in a humidified atmosphere at 37 °C and 5% CO_2 overnight. The next day, after lysis of the cells, absorbance was measured at 550 nm with a reference wavelength of 690 nm. Measurements were performed in duplicates. Results are given as percentage to the untreated control (=100%). Actinomycin D 10 μM was used as a control for reduction of cell viability.

2.7. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA and Dunnett's-Post-Test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of VLDL and fatty acids on intracellular calcium release upon AT1-receptor stimulation

Stimulation of the AT1R of the cells with angiotensin II results in a transient increase of intracellular calcium levels (Fig. 1 A). Preincubation of the cells with VLDL with concentrations of 5 μg/mL and above resulted in a decrease of calcium release from intracellular compartments upon angiotensin II stimulation compared to the untreated control (Fig. 1 A and B). Incubation with 450 μM stearic acid lead to a decrease of the intracellular calcium release upon angiotensin II stimulation, whereas 450 μM oleic acid resulted in an increase in the calcium release compared to the untreated control (Fig. 1C and D).

3.2. Effects of VLDL and fatty acids on the fluorescence ratio upon saturation of fura-2 with calcium

By adding an excess of extracellular calcium and the ionophore ionomycin, large amounts of extracellular calcium enters the cell and saturates the intracellular available fura-2. Hence, the fluorescence ratio reaches its maximal obtainable value (Fig. 2 A). Surprisingly, similar to the experiment with angiotensin II, preincubation of the cells with increasing VLDL concentrations resulted in a marked decrease of the maximal fluorescence ratio upon addition of high calcium and ionomycin solution. In cells exposed to VLDL concentrations of 5 μM and above, the maximum fluorescence ratio does not even exceed the peak maximum of the ratio seen after stimulation with angiotensin II (Fig. 2 A and B). Stearic acid or oleic acid did not result in an alteration of the maximal fluorescence ratio (Fig. 2C and D).

3.3. Effect of lipids on the amount of intracellular Fura-2

Ratiometric indicators change their fluorescence spectra with changing calcium concentrations, except for the wavelength of 360 nm, the isosbestic point, where the fluorescence of fura-2 is independent of the amount of bound calcium. Therefore, the fluorescence at 360 nm depends only of the amount of indicator in the cell. VLDL reduced dose-dependently the fluorescence at 360 nm (Fig. 3). Pretreatment with stearic or oleic acid did not change the fluorescence in the isosbestic point (Fig. 3).

3.4. Effects of lipids on cell vitality

Due to the unexpected results in the calcium assay, we assessed cytotoxic effects and metabolic activity after incubation with the different lipids. Damaged cells release LDH from the cytoplasm, which can be measured in the cell culture supernatant [16]. Only 30 μg/mL VLDL increased the LDH in the supernatant slightly but not statistically significant (39.97 ± 9.8 with VLDL 30 μg/mL versus 28.91 ± 6.7 in untreated control). Neither oleic nor stearic acid did induce LDH-release compared to the untreated control (Fig. 4 A).

Cell viability and metabolic activity was measured by the reduction of a tetrazolium salt in viable cells. Pretreatment of cells with VLDL dose-dependently reduced the viability in the cells, starting with 5 μg/mL VLDL. Stearic acid reduced the viability at a

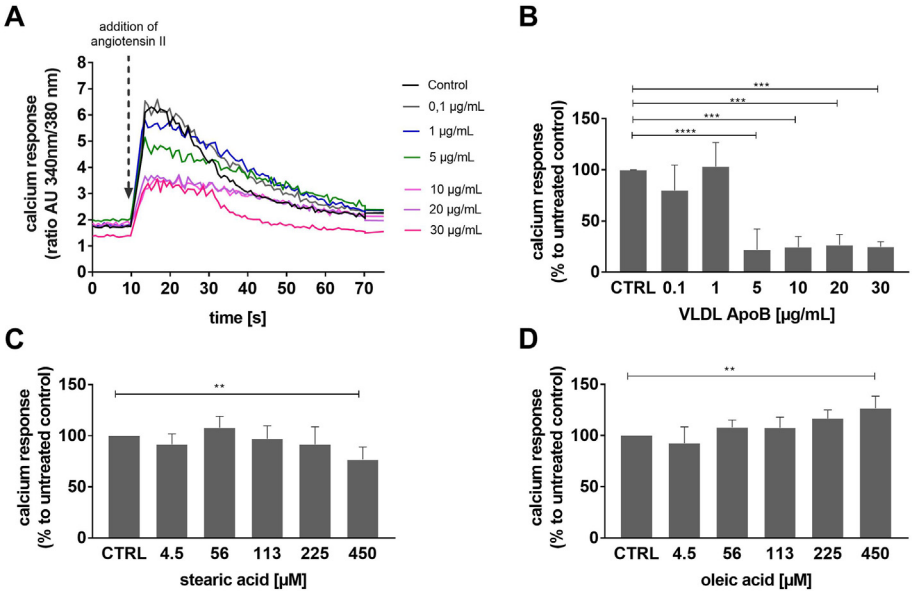

Fig. 1. Intracellular calcium response triggered by angiotensin II 2nM A) and B) in cells preincubated with different concentrations of VLDL C) and D) in cells preincubated with different concentrations of the fatty acid stearic acid or oleic acid respectively. A) shows a representative example of the calculated ratios over the time course of the experiment. Bars represent the maximum of the peak of the fluorescence ratio of 340/380 nm after stimulation with angiotensin II subtracted by the baseline of the ratio before stimulation. Data are given as mean ± SD (VLDL n = 3 and fatty acid each n = 5) in percent of the untreated control (without VLDL or fatty acid). Each column was tested against untreated control with one-way-ANOVA, followed by Dunnetts post-hoc-test. ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05.

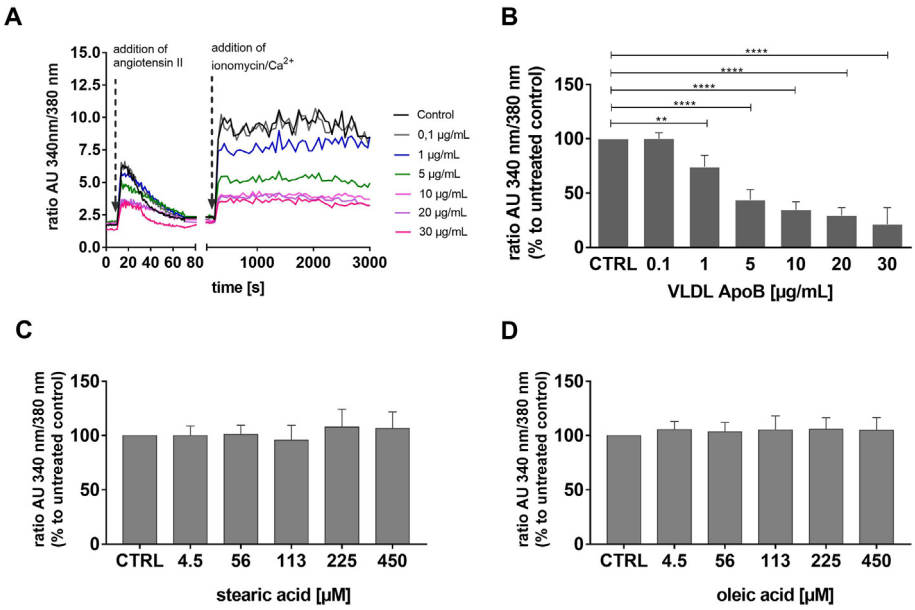

Fig. 2. Ratios after addition of the calcium ionophore ionomycin and extracellular excess of Ca^{2+} (representing saturation of intracellular fura-2 with calcium) A) and B) in cells preincubated with different concentrations of VLDL, C) and D) in cells preincubated with different concentrations of the fatty acid stearic acid or oleic acid respectively. A) shows a representative example of different courses of the fluorescence ratio in CHO-AT1 cells preincubated for 24 h with different concentrations of VLDL upon stimulation with angiotensin II (left arrow), and addition of ionomycin and excess of calcium (right arrow). Bars represent the ratios after addition of ionomycin and calcium subtracted by the baseline before the experiment. Data are given as mean ± SD (VLDL n = 4, fatty acid n = 8 each) in percent of the untreated control (without VLDL or fatty acid). Each column was tested against untreated control with one-way-ANOVA, followed by Dunnetts post-hoc-test. ****p < 0.0001; ***p < 0.001; **p < 0.01.

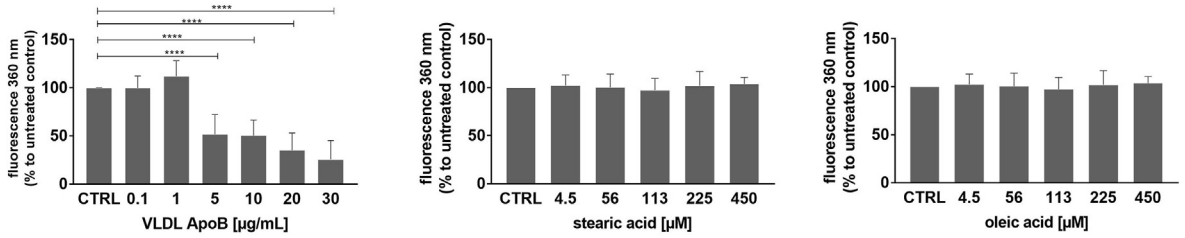

Fig. 3. Measured fluorescence at 360 nm (in arbitrary units) in cells incubated with VLDL, stearic acid or oleic acid as indicated. Data are given as mean ± SD (VLDL n = 12, fatty acid each n = 5) in percent to the untreated control (without VLDL or fatty acid). Each column was tested against the untreated control with one-way-ANOVA, followed by Dunnetts post-hoc-test. ****p < 0.0001.

concentration of 450 µmol, whereas oleic acid did not have any effect on cell viability (Fig. 4 B).

4. Discussion

Studies have shown, that cholesterol-rich lipoprotein LDL influences the activity of the AT1-Receptor [10,17]. In our work, we aimed at investigating the effects of another lipoprotein, the triglyceride-rich lipoprotein VLDL, and the fatty acids stearic acid (C18:0) and oleic acid (C18:1) on AT1R activity as measured by intracellular calcium release through the G_q-coupled receptor.

Measurement of intracellular calcium with calcium-sensitive dyes is a widely employed technique. Especially ratiometric indicators like fura-2 are widely used, as the change in the ratio of fluorescence, e.g. in the case of fura-2, measured at 340 nm and 380 nm is evaluated, and therefore confounders like dye concentration, dye leakage, cell number etc. should cancel themselves out [4]. There are several ways of reporting the results: Besides reporting blunt changes in the ratios, intensities could be normalized to baseline intensity (R/R_0) or calibrated to make numerical estimates of intracellular $[Ca^{2+}]_i$ concentrations [4]. But also these more sophisticated evaluation protocols have limitations: Reporting R/R_0 may lead to a slight underestimation of the "real" calcium level [4]. Henke et al. [18] reported underestimation of intracellular calcium concentrations derived from the classical calcium calibration approach by Grynkiewicz et al. [19]. Tiger and Fowler [20] conclude, that the calibration induces errors in the calcium measurements and that reporting only of the ratio yields more accurate results. However, in practice calibration is seldom performed.

For our purpose, namely to compare the impact of increasing concentrations of different lipid species on the receptor activity, measured by calcium release, calculation of absolute calcium concentrations does not add a significant additional information. Therefore, we used relative changes of ratios compared to respective untreated controls.

Preincubation with triglyceride-rich lipoprotein VLDL resulted in a significant decrease of calcium release from intracellular stores after stimulation with angiotensin II in CHO-AT1 cells. Stearic acid at a concentration of 450 µM did reduce the calcium response as well, whereas oleic acid did increase the calcium response.

However, in order for the evaluation of these fluorescence ratios within different samples to be truly meaningful, R_{max} should be similar in all investigated samples [19].

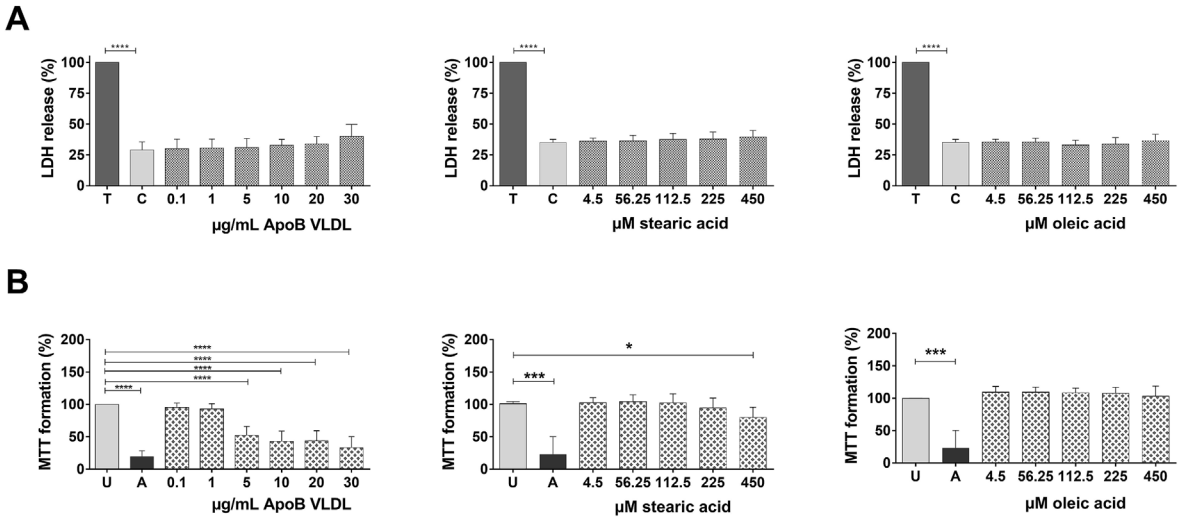

Fig. 4. Effects of different concentrations of lipids on cellular metabolism. **A)**: Cytotoxic effects of different concentrations of VLDL, stearic acid and oleic acid were quantified by measuring LDH in the supernatant. Data are given as mean ± SD (VLDL n = 4, fatty acid each n = 7). Triton-100 served as positive control of LDH-release and was set to 100%. Each column was tested against the untreated control (C) with one-way-ANOVA, followed by Dunnetts post-hoc-test. LDH-release by Triton-100 was statistically significant versus untreated control (****p < 0.0001). **B)**: Effects of different concentrations of VLDL, stearic acid and oleic acid on cell viability were quantified by measurement of formazan conversion with the MTT assay. Data are given as mean ± SD (VLDL n = 8, fatty acid each n = 8). Actinomycin D (A) served as positive control, the untreated control (U) was set to 100%. Each column was tested against untreated control (U) with one-way-ANOVA, followed by Dunnetts post-hoc-test. ****p < 0.0001; ***p < 0.001.

To reach $R_{max,}$ the indicator has to be completely saturated with calcium ions [4]. According to the equation of Grynkiewicz, intracellular calcium concentration is mainly a function of the ratio at any given time point (R), the ratio R_{min} (ratio upon zero calcium) and the ratio R_{max}.

$$Ca^{2+} = K_d \times \left[\frac{R - R_{min}}{R_{max} - R}\right] \times \left[\frac{F_{min}^{380}}{F_{max}^{380}}\right] \quad (1)$$

Equation (1) The Equation by Grynkiewicz describes the calculation of the intracellular calcium concentration derived from the ratios and the fluorescence measurement at wavelengths 340 and 380 nm at any given time point. K_d: dissociation constant of fura-2; R: ratio derived from measured fluorescence at wavelength 340 nm/380 nm at any given time point; R_{max}: Ratio upon complete saturation of intracellular fura-2 with Ca^{2+}; R_{min}: Ratio of fura-2 when completey free of Ca^{2+}

To estimate reasonable calcium concentrations, R_{max} should be well above the ratio obtained upon stimulation with angiotensin II, otherwise, the term $(R_{max}\text{-}R)$ approaches zero or would give negative values, which renders the result of the formula meaningless. We checked for this assumptions by measuring R_{max} in our experimental setting. Surprisingly, we did see a marked, dose-dependend reduction of R_{max} in cells incubated with VLDL, but not with the fatty acids. Additionally, in the case of VLDL, R_{max} did not exceed the maximal peak height after stimulation with angiotensin II, which means that the transient calcium release saturates the intracellular available fura-2. That means, that an increased calcium release can not be detected, because there is no available indicator to bind this amount of calcium.

To check, if the reduction of R_{max} is a result of a reduced amount of available intracellular fura-2, we measured the fluorescence at 360 nm, the isosbestic point. Fluorescence in the isosbestic point is independent of the calcium concentration [19] and is therefore indicative on the indicator concentration. Incubation with VLDL reduced the fluorescence at the isosbestic point (360 nm) in a dose-dependent manner. We concluded therefore, that the observed reduction in the calcium release after stimulation with angiotensin II was rather due to a limitation of the available fura-2 amount in the cells, than due to alterations in receptor activity. Neither stearic nor oleic acid showed any differences in the fluorescence at 360 nm at any concentration. Measuring only the ratios after stimulation with angiotensin II may have led to an artifact, proposing false effects of VLDL on AT1R activity. If we did not check for R_{max} and the fluorescence at 360 nm, we would not have been aware of this problem in our assay system.

The reason of the reduced intracellular amount of fura-2 in the cells is unclear. VLDL taken up by the cells via endocytosis are biochemically broken down into their components which may enter cellular metabolism. As VLDL themselves are not present during the actual fluorescence measurements, a direct effect through quenching or interfering autofluorescence is not likely.

Lipoproteins and fatty acids have cytotoxic effects in vitro [21–25]. A prerequisite for reliable fura-2-measurements in the cell is sufficient loading and complete de-esterification to obtain an adequate amount of free fura-2 in the cells [19,26]. Active esterases are important for fura-2-AM cleavage and subsequent fura-2 availability. Incubation with VLDL reduced the ability to convert the tetrazolium salt in the MTT assay and thus metabolic activity, while not leading to LDH release, typically observed by celltoxicity. It may be possible, that VLDL similarly hampers the activity of intracellular esterases and reduces the cleavage into fura-2. As no significant leakage of LDH in the supernatant was seen with neither of the compounds, it seems unlikely, that cleaved fura-2 exits the cell through a damaged and leaky cell membrane. VLDL releases primarily fatty acids [21] and membrane fluidity is altered by fatty acids [27,28]. The released fatty acids may alter membrane properties and reduce the diffusion of the fura-2-AM into the cells.

5. Conclusion

We show that incubation of cells with VLDL hampers calcium measurements with fura-2 even when VLDL is not present anymore at the time of the calcium measurement. The alterations of the cells by incubation with certain compounds may have detrimental effects on the assay system. The advantage of the ratiometric calcium

measurement is surely the robustness to diverging fura-2 concentration in different samples. Nevertheless, a very pronounced reduction of intracellular fura-2 may still render the measurements meaningless. We strongly recommend a measurement at the isosbestic point for each sample to check for adequate fura-2 loading, when preincubating the cells with compounds of toxic potential, as revealed by MTT assay. Otherwise, effects could be misinterpreted and erroneous conclusions may be drawn.

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Author contributions

CC and JK planned and conducted the experiments and evaluated the data. GP, UM, IM, and KW contributed to the planning of the experiments and evaluation of the data. CC wrote the initial manuscript draft. CC, JK, UM, KW and GP contributed to the development of the manuscript draft and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] S.J. Charlton, G. Vauquelin, Elusive equilibrium: the challenge of interpreting receptor pharmacology using calcium assays, Br. J. Pharmacol. 161 (2010) 1250–1265, https://doi.org/10.1111/j.1476-5381.2010.00863.x.
[2] M.A. Carrasco, C. Hidalgo, Calcium microdomains and gene expression in neurons and skeletal muscle cells, Cell Calcium 40 (2006) 575–583, https://doi.org/10.1016/j.ceca.2006.08.021.
[3] S. Orrenius, P. Nicotera, B. Zhivotovsky, Cell death mechanisms and their implications in toxicology, Toxicol. Sci. 119 (2011) 3–19, https://doi.org/10.1093/toxsci/kfq268.
[4] J.P.Y. Kao, G. Li, D.A. Auston, Practical aspects of measuring intracellular calcium signals with fluorescent indicators, Methods Cell Biol. 99 (2010) 113–152, https://doi.org/10.1016/B978-0-12-374841-6.00005-0.
[5] M. de Gasparo, K.J. Catt, T. Inagami, J.W. Wright, T. Unger, International union of pharmacology. XXIII. The angiotensin II receptors, Pharmacol. Rev. 52 (2000) 415–472.
[6] A. Nguyen, Dinh Cat, R.M. Touyz, Cell signaling of angiotensin II on vascular tone: novel mechanisms, Curr. Hypertens. Rep. 13 (2011) 122–128, https://doi.org/10.1007/s11906-011-0187-x.
[7] N. Sattar, J.R. Petrie, A.J. Jaap, The atherogenic lipoprotein phenotype and vascular endothelial dysfunction, Atherosclerosis 138 (1998) 229–235.
[8] G. Nickenig, O. Jung, K. Strehlow, O. Zolk, W. Linz, B.A. Schölkens, M. Böhm, Hypercholesterolemia is associated with enhanced angiotensin AT1-receptor expression, Am. J. Physiol. 272 (1997) H2701–H2707.
[9] G. Nickenig, S. Wassmann, M. Bohm, Regulation of the angiotensin AT1 receptor by hypercholesterolaemia, Diabetes Obes. Metabol. 2 (2000) 223–228.
[10] R.A. Catar, G. Müller, J. Heidler, G. Schmitz, S.R. Bornstein, H. Morawietz, Low-density lipoproteins induce the renin-angiotensin system and their receptors in human endothelial cells, Horm. Metab. Res. 39 (2007) 801–805, https://doi.org/10.1055/s-2007-991158.
[11] M. Asai, K. Takeuchi, S. Uchida, T. Urushida, H. Katoh, H. Satoh, S. Yamada, H. Hayashi, H. Watanabe, Misinterpretation of the effect of amlodipine on cytosolic calcium concentration with fura-2 fluorospectrometry, Naunyn-Schmiedeberg's Arch. Pharmacol. 377 (2008) 423–427, https://doi.org/10.1007/s00210-007-0243-x.
[12] R.C. Paudel, S. Kiviluoto, J.B. Parys, G. Bultynck, Resveratrol is not compatible with a Fura-2-based assay for measuring intracellular Ca2+ signaling, Biochem. Biophys. Res. Commun. 450 (2014) 1626–1630, https://doi.org/10.1016/j.bbrc.2014.07.049.
[13] P. Santofimia-Castaño, G.M. Salido, A. Gonzalez, Interferences of resveratrol with fura-2-derived fluorescence in intracellular free-Ca2+ concentration determinations, Cytotechnology (2015), https://doi.org/10.1007/s10616-015-9898-1.
[14] J.E. Schaffer, Lipotoxicity: when tissues overeat, Curr. Opin. Lipidol. 14 (2003) 281–287, https://doi.org/10.1097/00041433-200306000-00008.
[15] K. Winkler, B. Wetzka, M.M. Hoffmann, I. Friedrich, M. Kinner, M.W. Baumstark, H.P. Zahradnik, H. Wieland, W. März, Triglyceride-rich lipoproteins are associated with hypertension in preeclampsia, J. Clin. Endocrinol. Metab. 88 (2003) 1162–1166, https://doi.org/10.1210/jc.2002-021160.
[16] C. Korzeniewski, D.M. Callewaert, An enzyme-release assay for natural cytotoxicity, J. Immunol. Methods 64 (1983) 313–320, https://doi.org/10.1016/0022-1759(83)90438-6.
[17] G. Nickenig, A. Sachinidis, F. Michaelsen, M. Böhm, S. Seewald, H. Vetter, Upregulation of vascular angiotensin II receptor gene expression by low-density lipoprotein in vascular smooth muscle cells, Circulation 95 (1997) 473–478.
[18] W. Henke, C. Cetinsoy, K. Jung, S. Loening, Non-hyperbolic calcium calibration curve of Fura-2: implications for the reliability of quantitative Ca2+ measurements, Cell Calcium 20 (1996) 287–292.
[19] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca2+ indicators with greatly impoved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450, 3838314.
[20] C.J. Fowler, G. Tiger, Calibration of Fura-2 signals introduces errors into measurement of thrombin-stimulated calcium mobilisation in human platelets, Clin. Chim. Acta 265 (1997) 247–261.
[21] L. Wang, R. Gill, T.L. Pedersen, L.J. Higgins, J.W. Newman, J.C. Rutledge, Triglyceride-rich lipoprotein lipolysis releases neutral and oxidized FFAs that induce endothelial cell inflammation, J. Lipid Res. 50 (2009) 204–213, https://doi.org/10.1194/jlr.M700505-JLR200.
[22] I.J. Goldberg, K.E. Bornfeldt, Lipids and the endothelium: bidirectional interactions, Curr. Atherosclerosis Rep. 15 (2013) 365, https://doi.org/10.1007/s11883-013-0365-1.
[23] L.L. Listenberger, X. Han, S.E. Lewis, S. Cases, R. V Farese, D.S. Ory, J.E. Schaffer, Triglyceride accumulation protects against fatty acid-induced lipotoxicity, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 3077–3082, https://doi.org/10.1073/pnas.0630588100.
[24] K. Staiger, H. Staiger, C. Weigert, C. Haas, H.U. Haring, M. Kellerer, Saturated, but not unsaturated, fatty acids induce apoptosis of human coronary artery endothelial cells via nuclear factor-kappaB activation, Diabetes 55 (2006) 3121–3126, https://doi.org/10.2337/db06-0188.
[25] A. Peter, C. Weigert, H. Staiger, K. Rittig, A. Cegan, P. Lutz, F. Machicao, H.-U. Häring, E. Schleicher, Induction of stearoyl-CoA desaturase protects human arterial endothelial cells against lipotoxicity, Am. J. Physiol. Endocrinol. Metab. 295 (2008) E339–E349, https://doi.org/10.1152/ajpendo.00022.2008.
[26] M.W. Roe, J.J. Lemasters, B. Herman, Assessment of Fura-2 for measurements of cytosolic free calcium, Cell Calcium 11 (1990) 63–73, https://doi.org/10.1016/0143-4160(90)90060-8.
[27] A. Raynor, P. Jantscheff, T. Ross, M. Schlesinger, M. Wilde, S. Haasis, T. Dreckmann, G. Bendas, U. Massing, Saturated and mono-unsaturated lysophosphatidylcholine metabolism in tumour cells: a potential therapeutic target for preventing metastases, Lipids Health Dis. 14 (2015) 69, https://doi.org/10.1186/s12944-015-0070-x.
[28] K. Hac-Wydro, P. Wydro, The influence of fatty acids on model cholesterol/phospholipid membranes, Chem. Phys. Lipids 150 (2007) 66–81, https://doi.org/10.1016/j.chemphyslip.2007.06.213.