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Marine omega-3 fatty acids prevent myocardial insulin resistance and metabolic remodeling as induced experimentally by high insulin exposure

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[1]*Cardiovascular Research Group, Department of Medical Biology, Health Sciences Faculty, UiT The Arctic University of Norway, Tromsø, Norway;* [2]*Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, the Netherlands; and* [3]*Bioceros BV, Utrecht, the Netherlands*

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Franekova V, Angin Y, Hoebers NT, Coumans WA, Simons PJ, Glatz JF, Luiken JJ, Larsen TS. Marine omega-3 fatty acids prevent myocardial insulin resistance and metabolic remodeling as induced experimentally by high insulin exposure. *Am J Physiol Cell Physiol* 308: C297–C307, 2015. First published December 4, 2014; doi:10.1152/ajpcell.00073.2014.—Insulin resistance is an important risk factor for the development of several cardiac pathologies, thus advocating strategies for restoring insulin sensitivity of the heart in these conditions. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs), mainly eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), have been shown to improve insulin sensitivity in insulin-sensitive tissues, but their direct effect on insulin signaling and metabolic parameters in the myocardium has not been reported previously. The aim of this study was therefore to examine the ability of EPA and DHA to prevent insulin resistance in isolated rat cardiomyocytes. Primary rat cardiomyocytes were made insulin resistant by 48 h incubation in high insulin (HI) medium. Parallel incubations were supplemented by 200 μM EPA or DHA. Addition of EPA or DHA to the medium prevented the induction of insulin resistance in cardiomyocytes by preserving the phosphorylation state of key proteins in the insulin signaling cascade and by preventing persistent relocation of fatty acid transporter CD36 to the sarcolemma. Only cardiomyocytes incubated in the presence of EPA, however, exhibited improvements in glucose and fatty acid uptake and cell shortening. We conclude that ω-3 PUFAs protect metabolic and functional properties of cardiomyocytes subjected to insulin resistance-evoking conditions.

omega-3 PUFAs; cardiac metabolism; insulin resistance; CD36; adipose triglyceride lipase; sarcomere shortening

AN ELEVATED SUPPLY of lipids in obesity and type 2 diabetes leads to alterations of myocardial substrate metabolism, manifested insulin resistance with reduced glucose utilization, and increased long-chain fatty acid (LCFA) utilization (2, 3, 39). Thus isolated cardiomyocytes from various diabetic/obese rodent models show impaired insulin signaling, including reduced activation of protein kinase B (Akt/PKB), reduced glucose transporter 4 (GLUT4) translocation from intracellular compartments to the sarcolemma, as well as impaired insulin-induced glucose uptake (10, 35, 37). Glucose and LCFA are the major substrates for the heart, and GLUT4 and CD36 the major substrate transporters, which are regulated by reversible insulin-induced translocation (48). Notably, increased LCFA supply to cardiomyocytes will evoke persistent relocation of CD36 from intracellular stores to the sarcolemma, followed by chronically elevated LCFA uptake and lipid accumulation, eventually resulting in insulin resistance (5, 36). Additionally, sustained hyperinsulinemia itself has also been shown to chronically stimulate CD36 translocation in cardiomyocytes and, consequently, lead to insulin resistance in a very similar manner to cardiomyocytes exposed to LCFA oversupply, as reported previously (5). We also found that insulin-resistant cardiomyocytes exhibit contractile dysfunction (reduced cell shortening) (5).

Numerous in vivo studies in rodent models of obesity and diabetes have clearly shown that diets enriched in EPA and/or DHA improve whole body insulin resistance (15, 22, 42, 45). However, findings on the effect of ω-3 PUFAs on human insulin resistance are controversial, since some studies reported no clear improvements, while others showed positive effects on insulin sensitivity (4, 13, 27, 31, 44, 47, 51). The explanation for these controversial findings remains unclear but may be attributable to differences in health status, medication, diet, and compliance among the subjects, as well as differences in duration and dose of ω-3 PUFAs administration. Regardless of the inconsistencies, a number of in vivo and in vitro studies show that intake of ω-3 PUFAs may lead to reduction of insulin resistance due to their effect on expression and activity of glucose metabolizing enzymes, decreased expression of lipogenesis enzymes, increased fatty acid oxidation, or the ability to inhibit inflammation (13, 21, 32). However, despite these known benefits, the direct effect of EPA and DHA on insulin signaling as well as on metabolic and functional parameters of the heart has not been assessed yet.

To test the putative preventive effect of EPA and DHA on myocardial insulin resistance, primary rat cardiomyocytes were made insulin resistant by long-term exposure to a medium containing high concentrations of insulin, as previously described (5, 6). Cells cultured under these conditions were coincubated with EPA and DHA complexed to albumin. Insulin sensitivity was evaluated by examining the response to acute insulin exposure on glucose and LCFA uptake, as well as phosphorylation of selected proteins in the insulin signaling cascade. In addition, the effect of EPA and DHA on the presence of fatty acid transport protein CD36 in the sarcolemma was examined by confocal microscopy. Of interest, several studies in rat and mouse models of pressure overload-induced heart failure have reported that intake of EPA and DHA prevents left ventricular remodeling and contractile dysfunction (8, 12, 34). Therefore, we also examined the effect of EPA and DHA on myocardial cell shortening.

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MATERIALS AND METHODS

Medium M199 and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Phloretin, laminin, creatine monohydrate, carnitine hydrochloride, taurine, insulin, palmitic acid, EPA, and DHA were purchased from Sigma Aldrich (St. Louis, MO). Collagenase type II was from Worthington (Freehold, NJ). BSA (essentially fatty acid free, fraction V) was derived from MP Biomedicals (Irvine, CA) or from Sigma Aldrich (St. Louis. MO). [1-^{3}H]deoxyglucose and [1-^{14}C]palmitate were obtained from GE Healthcare (Piscataway, NJ). The anti-CD36 monoclonal antibody (clone CRF D2717) was from BD Biosciences (San Jose, CA) and was provided by Bioceros BV (Utrecht, the Netherlands).

Experimental animals. Male Lewis rats were purchased from Charles River Laboratories (Boston, MA) and were used for isolation of cardiomyocytes. All animals were fed ad libitum. All procedures were approved by the Experimental Animal Committee of Maastricht University, Maastricht, the Netherlands.

Cardiomyocytes isolation and culturing. Isolation of rat adult cardiomyocytes (Lewis rats, 200–250 g, 2–3 mo of age) was performed by using a Langendorff perfusion system according to the procedure described previously (29) with the only difference being sterile conditions that were taken into account to enable subsequent culturing. A modified Krebs-Ringer buffer (MKR) containing 1.17 M NaCl, 26 mM KCl, 12 mM KH_2PO_4, 12 mM $MgSO_4 \cdot 7H_2O$, 100 mM $NaHCO_3$, and 100 mM HEPES with pH adjusted to 7.55 was equilibrated with 95% O_2-5% CO_2 gas phase and used for isolation and experiments with cardiomyocytes.

After isolation of cardiomyocytes, 100,000 cells/ml were seeded in MKR buffer supplemented with 0.45% (wt/vol) BSA on laminin-coated six-well plates (9.6 cm^2). For sarcomere shortening and the surface CD36 measurements cells were seeded on 35-mm-high dishes with an elastic surface (Ibidi GmBH, Munich, Germany). After 90 min adhesion, the medium was replaced with control medium (medium M199 supplemented with 5 mM creatine monohydrate, 3.2 mM carnitine hydrochloride, 3.1 mM taurine, 100 units/ml penicillin, 10 mg/ml streptomycin, and 20 μM palmitate), HI medium (control medium supplemented with 100 nM insulin), or fatty acid medium (HI medium supplemented with 200 μM EPA or DHA). For cardiomyocytes cotreated with anti-CD36 (clone CRF D2717) an antibody concentration 0.415 μg/ml was used. Fatty acids were bound to BSA (essentially fatty acid free) in a ratio 4:1. BSA was used as fatty acid free control added to the control medium to avoid interference from vehicle. Cardiomyocytes were kept in 5% CO_2 at 37°C for 48 h. Medium was refreshed after 24 h of seeding. Following 48 h of culturing, cells were washed and allowed to recover for 30 min in MKR buffer supplemented with 0.45% (wt/vol) BSA and 1 mM $CaCl_2$ prior to start of 25 min incubation with or without insulin (100 nM).

Measurement of substrate uptake. The uptake of [1-^{3}H]deoxyglucose and of [1-^{14}C]palmitate (in complex with BSA) was measured in cultured cardiomyocytes. During 25 min treatment with/without insulin (100 nM), trace amounts of [1-^{3}H]deoxyglucose and [1-^{14}C]palmitate were added to the medium for the last 10 min of incubation. Thereafter, cells were washed twice on ice with MKR buffer supplemented with 1 mM $CaCl_2$ and 0.2 mM phloretin, and then lysed in sample buffer [40% (vol/vol) glycerol, 0.25 M Tris, and 1 M DTT] followed by assessment of radioactivity in scintillation fluid (Opti-Fluor; PerkinElmer, Waltham, MA).

Detection of phosphorylation of enzymes within the insulin signaling network. Following a 25 min treatment with/without insulin (100 nM) cardiomyocytes were lysed in sample buffer and used for protein detection. Approximately 20 μg per sample was used for SDS-PAGE followed by Western blotting.

Primary antibodies specific to Akt, phospho-Ser473-Akt, phospho-Thr172-AMPKα, phospho-Ser9-glycogen synthase kinase 3β (GSK3β), mammalian target of rapamycin (mTOR), phospho-Ser2448-mTOR, p70 S6K, phospho-Thr389-p70 S6K, and adipose triglyceride lipase (ATGL) purchased from Cell Signaling Technology (Denver, CO), phospho-Ser79-ACC and phospho-Thr642-AS160 (AS160-Akt substrate of 160 kDa) from Upstate Biotechnology (Millipore), GLUT4 and GLUT1 from Abcam (Cambridge, MA), and Caveolin 3 from BD Transduction Laboratories (Franklin Lakes, NJ) were used. The anti-CD36 MO25 antibody was a gift from N. N. Tandon (Thrombosis Research Laboratory, Otsuka Maryland Medicinal Laboratories Rockville, MD) and was used only for Western blotting. Western blot images were analyzed with a molecular imager (ChemiDoc XRS, Bio-Rad Laboratories) or Kodak imaging system (Kodak, Scientific Imaging Systems, Rochester, NY) and quantified with Quantity One (Bio-Rad Laboratories) or Kodak 1D Image Analysis Software (Kodak).

Quantitative real-time PCR. Primary rat cardiomyocytes cultured under different conditions were harvested after 48 h and used as the source for mRNA extraction using the Perfect Pure RNA Cultured Cell kit (5Prime, Norway) following the manufacturer's protocol. Rat RNA isolated from individual samples was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. Real-time quantitative PCR was performed using ABI PRISM 7900 HT Fast real-time thermal cycler (Applied Biosystems). Measurement of mRNA expression levels of ATGL and Acyl-CoA:diacylglycerol acyltransferase-1 (DGAT-1) was determined by SYBR Green master mix (Applied Biosystems) using the following rat primer pairs obtained from Sigma (Norway): ATGL forward (ggcctactgaaccaacccaa), reverse (agccactccaacaaacgcat); and DGAT-1 forward (gttcagctcagacagcggttt), reverse (catcaccacgcaccaattca). GAPDH served as the housekeeping reference gene for measuring the levels of the individual mRNA.

Sarcolemmal presence of CD36. Following 48 h of culturing, cells were washed twice with control medium, treated with primary antibody against CD36 (BD Biosciences) for 10 min at 37°C followed by incubation with FITC-labeled rabbit-anti-mouse IgA secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA). The viable cardiomyocytes were imaged using the Leica SP5 imaging platform in confocal mode (Leica Microsystems) with the emission filters optimized for FITC detection. Images were processed with ImageJ (NIH).

Measurement of sarcomere shortening. Following 48 h of culturing, cells were washed twice with control medium and subsequently analyzed for contractile function as previously described (18). Before the start of the measurements, cells were electrically prestimulated for 5 min with 1 Hz to reach a steady-state level for sarcomere shortening. Then cells were paced with bipolar pulses of 5 ms duration at 1 Hz. Under each experimental condition, data files were recorded of 10 consecutive beats for at least eight different cells. Sarcomere shortening was measured with IonOptix (Dublin, Ireland) and calculated using IonWizard (IonOptix).

Statistical analysis and data presentation. All data are presented as means ± SE. Statistical analyses were performed by one-way ANOVA followed by Tukey post test using GraphPad Prism Program (GraphPad Software, San Diego, CA). $P < 0.05$ was considered as significant.

RESULTS

Effect of EPA and DHA on glucose uptake. Insulin resistance was induced in primary rat cardiomyocytes during 48 h exposure to a medium containing high concentration (100 nM) of insulin (HI medium). In addition, cardiomyocytes incubated in HI medium were coincubated with 200 μM EPA or DHA. Cardiomyocyte insulin sensitivity was assessed by insulin-stimulated glucose uptake.

Cardiomyocytes cultured for 48 h in control medium (medium without HI) responded to acute (25 min) stimulation with insulin (100 nM) by a 3.4-fold increase in glucose uptake,

while glucose uptake was hardly increased by acute insulin stimulation in cardiomyocytes cultured in HI medium (insulin-resistant cells). Culturing of cardiomyocytes in HI medium in the presence of EPA elevated insulin-stimulated glucose uptake and thus prevented the loss of insulin sensitivity (Fig. 1*A*). Interestingly, basal glucose uptake was also significantly elevated in cardiomyocytes upon EPA exposure during 48 h of culturing in HI medium. Thus there was no difference in insulin-induced increment in glucose uptake between control and EPA-treated cardiomyocytes (Fig. 1*A*).

The effect of DHA on glucose uptake was tested in parallel experiments, and like EPA, DHA also caused an increase in basal glucose uptake in cardiomyocytes subjected to insulin resistance-evoking conditions. On the other hand, DHA treatment did not prevent the loss of insulin-stimulated glucose uptake in cardiomyocytes cultured in HI medium (Fig. 1*A*).

Figure 1*B* shows that the observed improvements in glucose uptake upon exposing cardiomyocytes to EPA or DHA were not associated with alterations in the total level of the glucose transporter GLUT1 or GLUT4.

Effect of EPA and DHA on insulin signaling pathway. A commonly used read-out of insulin resistance is decreased phosphorylation of enzymes in the canonical insulin signaling pathway. Insulin resistance upon 48 h culturing in HI medium was confirmed in cardiomyocytes by markedly lower phosphorylation of Akt/PKB (phospho-Ser473) and two of its downstream substrates, GSK3β (phospho-Ser9) and AS160 (phospho-Thr642-AS160), in response to acute (25 min) exposure to insulin (100 nM), compared with that of control cells (Fig. 2, *A* and *C–E*). Insulin resistance was also observed at the level of mTOR, given the decrease in phosphorylation of mTOR (phospho-Ser2448) and its direct target p70 S6K (phospho-Thr389) (Fig. 2, *B, F*, and *G*). Addition of EPA or DHA to the culture medium during the 48 h incubation with HI almost completely prevented the loss of insulin sensitivity at the level of Akt/PKB, since insulin-induced phosphorylation of Akt/PKB, GSK3β, and AS160 was largely retained (Fig. 2, *A* and *C–E*). However, insulin sensitivity was not preserved by EPA or DHA at the level of mTOR and p70 S6K phosphorylation (Fig. 2, *B, F*, and *G*). We also observed that exposure of HI medium-cultured cardiomyocytes to ω-3 PUFAs (especially EPA) caused a slight increase in basal phosphorylation of proteins involved in insulin signaling (Fig. 2, *A–G*). These changes in insulin signaling were not due to different loading or changes in total expression of Akt/PKB, mTOR, or p70 S6K (data not shown).

Effect of EPA and DHA on AMPK signaling. It is also disputed whether activation of AMP-activated protein kinase (AMPK) is involved in the beneficial effects of ω-3 PUFAs. Namely, mice treated with diet containing these fatty acids did not show any changes in the activity of myocardial AMPK (11), whereas ω-3 PUFAs have been shown to enhance AMPK activity in other tissues (16, 23, 26, 46). Hence, we assessed the phosphorylation level of AMPK and its downstream target, acetyl-CoA carboxylase (ACC). Culturing of cardiomyocytes in HI medium in the presence of EPA or DHA had, however, no significant effect on AMPK or ACC phosphorylation (Fig. 3). Hence, AMPK seems not to be involved in these beneficial actions of EPA and DHA.

Effect of EPA and DHA on fatty acid uptake, sarcolemmal presence of CD36, and fatty acid-metabolic enzymes. Cardiomyocytes cultured for 48 h in control medium responded to acute stimulation with insulin by a 1.4-fold increase in palmitate uptake. This response was lost in cardiomyocytes cultured under HI conditions, while inclusion of EPA (but not DHA) in the HI medium prevented the loss of insulin sensitivity (Fig. 4*A*). Furthermore, both EPA and DHA treatment caused a marked (>2-fold) stimulation of basal palmitate uptake in cardiomyocytes cultured under HI conditions. The changes in palmitate uptake were not associated with changes in the total

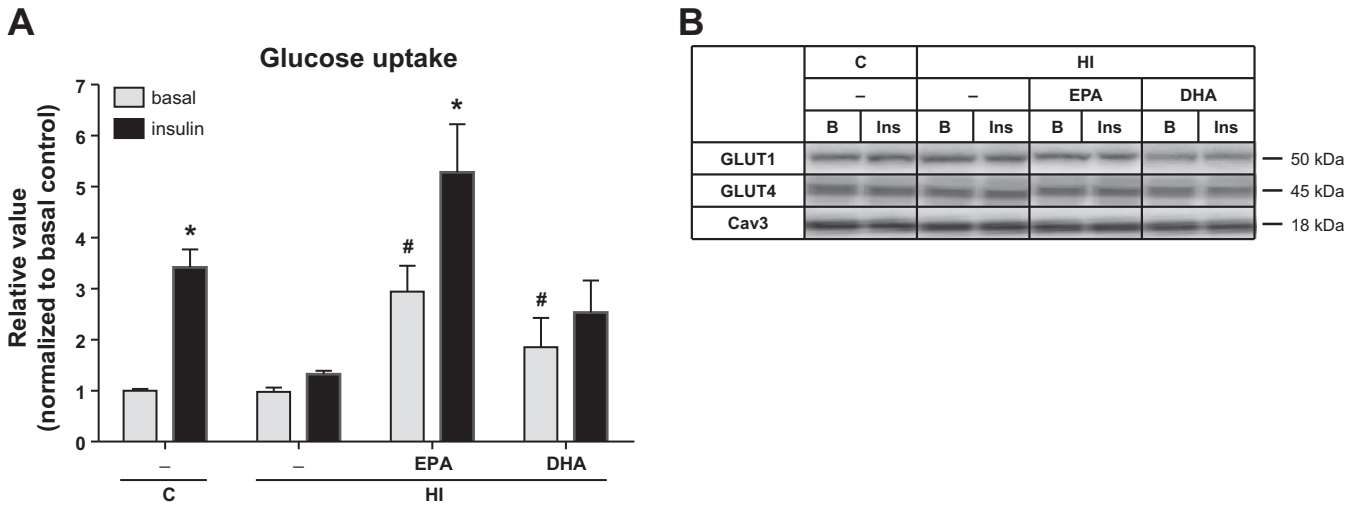
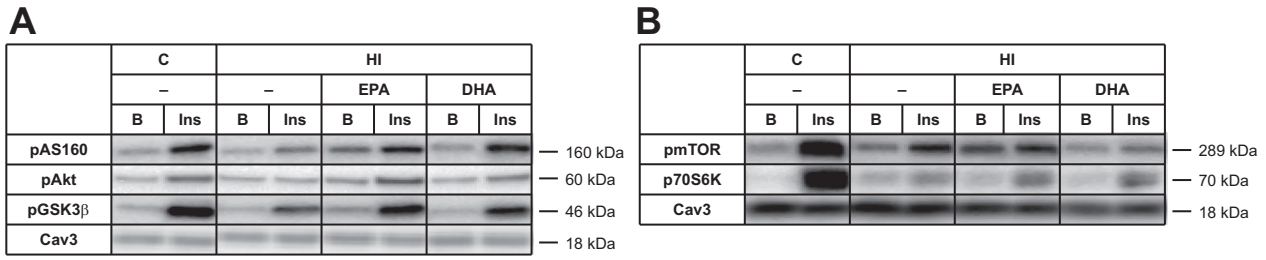
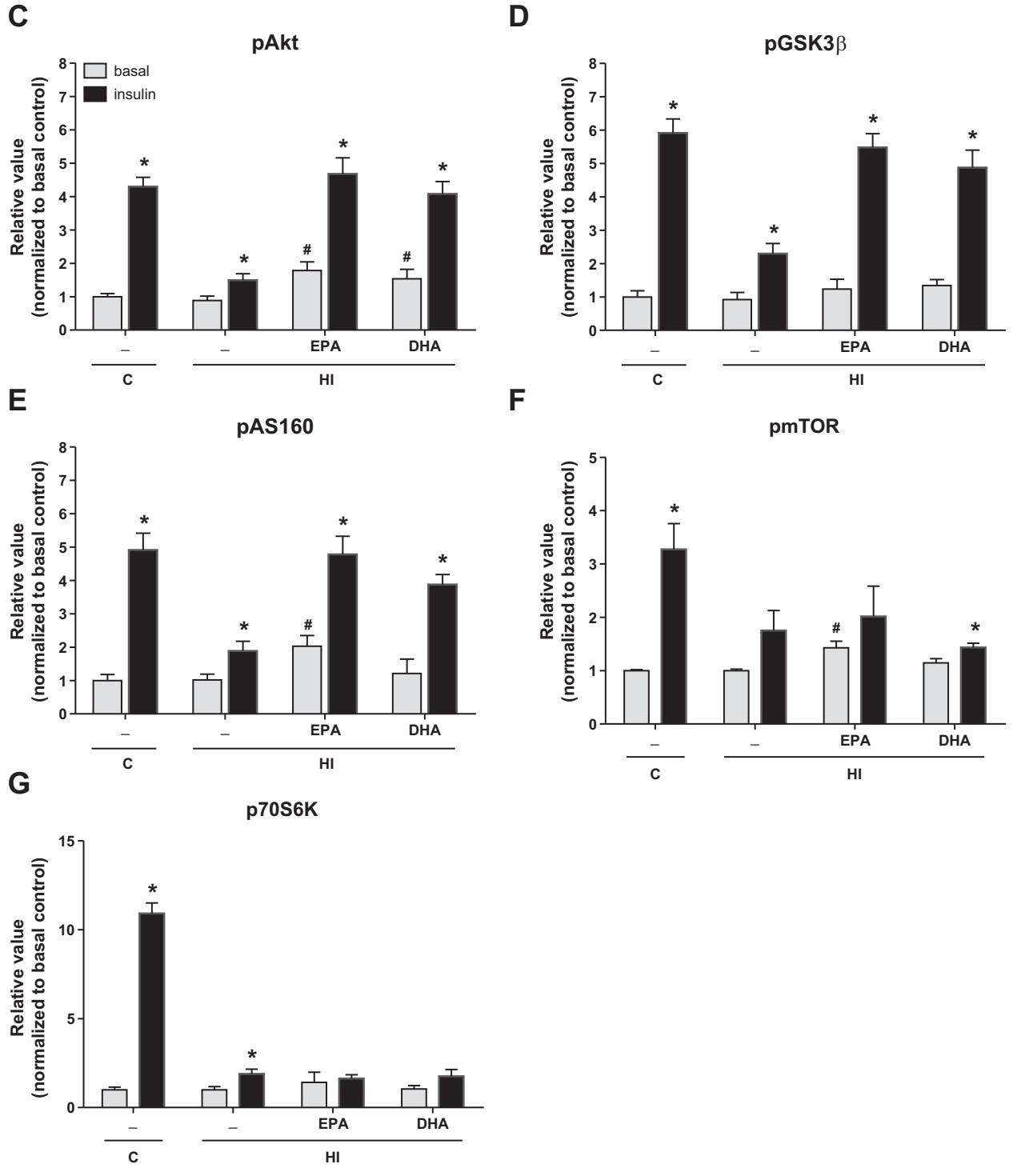


Fig. 1. Effect of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) on glucose uptake and total expression level of GLUT1 and GLUT4. Cardiomyocytes were preincubated for 48 h in control medium (C) or in medium supplied with 100 nM insulin (HI) in the absence or presence of 200 μM eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Thereafter, cells were washed and allowed to recover for 30 min prior to short-term (25 min) incubation with or without insulin (100 nM). For measurement of glucose uptake [^{3}H]deoxyglucose was added to medium for the last 10 min of incubation. *A*: glucose uptake data obtained from cardiomyocytes cultured in control or HI medium in the absence or presence of ω-3 PUFAs during basal and insulin-stimulated conditions. Results are represented as mean values ± SE ($n = 11$ for treatment with EPA, $n = 4$ for treatment with DHA). *$P < 0.05$ vs. corresponding basal value; #$P < 0.05$ vs. basal value in the absence of ω-3 PUFAs. *B*: representative Western blot analyses ($n = 4–6$) of total GLUT1 and GLUT4 in cardiomyocytes cultured in control or HI medium in the absence or presence of ω-3 PUFAs during basal (B) and insulin-stimulated conditions (Ins). Caveolin 3 (Cav3) was used as loading control.





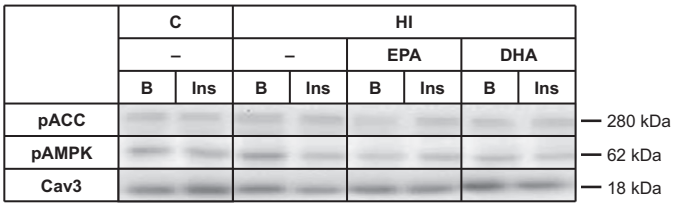


Fig. 3. Effect of ω-3 PUFAs on AMPK signaling. Cardiomyocytes were pretreated and stimulated with insulin as described in the legend of Fig. 1. Representative Western blot analyses ($n = 6$) of phospho-Thr172-AMPKα AMPK (pAMPK) and phospho-Ser79-ACC (pACC) in cardiomyocytes cultured in control (C) or high insulin (HI) medium in the absence or presence of ω-3 PUFAs during basal (B) and insulin-stimulated conditions (Ins). Caveolin 3 (Cav3) was used as loading control.

cellular content of the fatty acid transporter CD36 (Fig. 4*B*). We also examined the sarcolemmal abundance of CD36 by using immunofluorescence microscopy (Fig. 4, *C* and *D*). Cardiomyocytes cultured in HI medium exhibited a markedly elevated cell surface CD36 content. Persistent CD36 relocation was reversed in incubations with EPA or DHA.

We have shown earlier that the fatty acid transport function of CD36 can be blocked by specific anti-CD36 antibody (5). In line with this result, we found that coincubation of EPA with anti-CD36 antibody abolished both basal and insulin-stimulated palmitate uptake in cardiomyocytes, which might suggest that EPA exerts its insulin sensitizing action after having been taken up across the cell membrane through CD36 (Fig. 4*A*).

To obtain information about the effects of EPA and DHA on the metabolic fate of fatty acids in cardiomyocytes cultured under HI conditions, especially in relation to incorporation into myocellular lipid pools, we studied the effects of these treatments on expression of acyl-CoA:diacylglycerol acyltransferase-1 (DGAT-1), a key enzyme involved in triacylglycerol synthesis, and of ATGL, a key enzyme in triacylglycerol breakdown. mRNA expression of DGAT-1 was not different in any conditions (Fig. 5*A*). mRNA expression of ATGL was not different between control cells and cells cultured under HI conditions, but increased upon treatment with EPA (Fig. 5*B*). This stimulatory action of EPA on ATGL expression was confirmed at the protein level (Fig. 5, *C* and *D*). The presence of DHA had, however, no significant effect on ATGL expression (Fig. 5, *B–D*).

Effect of EPA and DHA on cardiomyocyte contractility (shortening). It has been previously shown that long-term exposure of primary rat cardiomyocytes to HI is associated with impaired peak sarcomere shortening (5), which was confirmed in the present study (Fig. 6). Here we investigated the effect of EPA and DHA on sarcomere shortening of cardiomyocytes cultured in HI medium for 48 h. EPA improved peak sarcomere shortening by 1.6-fold (Fig. 6). DHA also improved sarcomere shortening (1.5-fold); however, this effect did not reach statistical significance ($P > 0.05$; Fig. 6). Hence, EPA, and perhaps DHA, has a beneficial effect on contractile function of cardiomyocytes cultured under such insulin resistance-evoking conditions.

Effect of EPA in insulin-sensitive cardiomyocytes. For comparative reasons we assessed whether ω-3 PUFAs affected substrate uptake in normal cardiomyocytes (cultured without HI), using only EPA since it showed a greater scale of beneficial effects than DHA under HI conditions.

Like HI medium-cultured cardiomyocytes, control cells also showed elevated basal glucose uptake and a clear response to insulin following exposure to EPA (Fig. 7*A*). Basal fatty acid uptake was also increased in normal cardiomyocytes treated with EPA; however, these cells did not respond to acute insulin treatment (Fig. 7*B*). The changes in glucose and palmitate uptake were not associated with changes in the total cellular content of the glucose transporter GLUT1, GLUT4, or fatty acid transporter CD36 (data not shown).

Finally, just as in insulin-resistant cardiomyocytes, EPA treatment also decreased the plasma membrane level of CD36 (Fig. 8, *A* and *B*), increased ATGL expression (Fig. 8, *C–E*), did not alter DGAT-1 expression (data not shown), and improved the contractility in normal cardiomyocytes compared with control cells (Fig. 8*F*).

DISCUSSION

Dysregulation of cardiac substrate metabolism in conditions of insulin resistance (obesity and/or type 2 diabetes) eventually leads to functional deterioration of the heart. In the present study we investigated the effect of ω-3 PUFAs on insulin signaling and substrate uptake in cardiomyocytes cultured under insulin resistance-evoking conditions generated by a high concentration of insulin. The main finding was that inclusion of ω-3 PUFAs in the HI medium counteracted the development of insulin resistance. Apparently, the potential of EPA to prevent insulin resistance exceeded that of DHA.

Before elaborating on the potential beneficial effects of ω-3 PUFAs against the development of insulin resistance, it is worthwhile to mention that EPA has already clear metabolic and functional actions in normal insulin-sensitive cardiomyocytes. Specifically, we found that basal glucose and palmitate uptake were increased after treatment with this fatty acid. Aas et al. (1) also observed an increase in basal glucose and fatty acid uptake after treatment with EPA in human skeletal muscle cells. This observation could probably be explained in terms of a general anabolic effect of ω-3 PUFAs, in line with results reported by Smith et al. (43) showing increased muscle protein concentration in response to dietary intake of ω-3 PUFAs. However, in light of the observation that EPA or DHA did not increase p70 S6K phosphorylation, the activity of the anabolic master regulator mTOR appears not to be altered by these ω-3

Fig. 2. Effect of ω-3 PUFAs on insulin signaling. Cardiomyocytes were pretreated and stimulated with insulin as described in the legend of Fig. 1. *A* and *B*: representative Western blot analyses ($n = 4–10$) of phospho-Ser473-Akt (pAKT), phospho-Ser9-GSK3β (pGSK3β), phospho-Thr642-AS160 (pAS160), phospho-Ser2448-mTOR (pmTOR), and phospho-Thr389-p70 S6K (p70 S6K) in cardiomyocytes cultured in control (C) or high insulin (HI) medium in the absence or presence of ω-3 PUFAs during basal (B) and insulin-stimulated conditions (Ins). Caveolin 3 (Cav3) was used as loading control. *C–G*: quantification of the signals from Western blot analyses corrected for loading control. Results are represented as mean values ± SE ($n = 4–10$). $^*P < 0.05$ vs. corresponding basal value; $\#P < 0.05$ vs. basal value in the absence of ω-3 PUFAs.

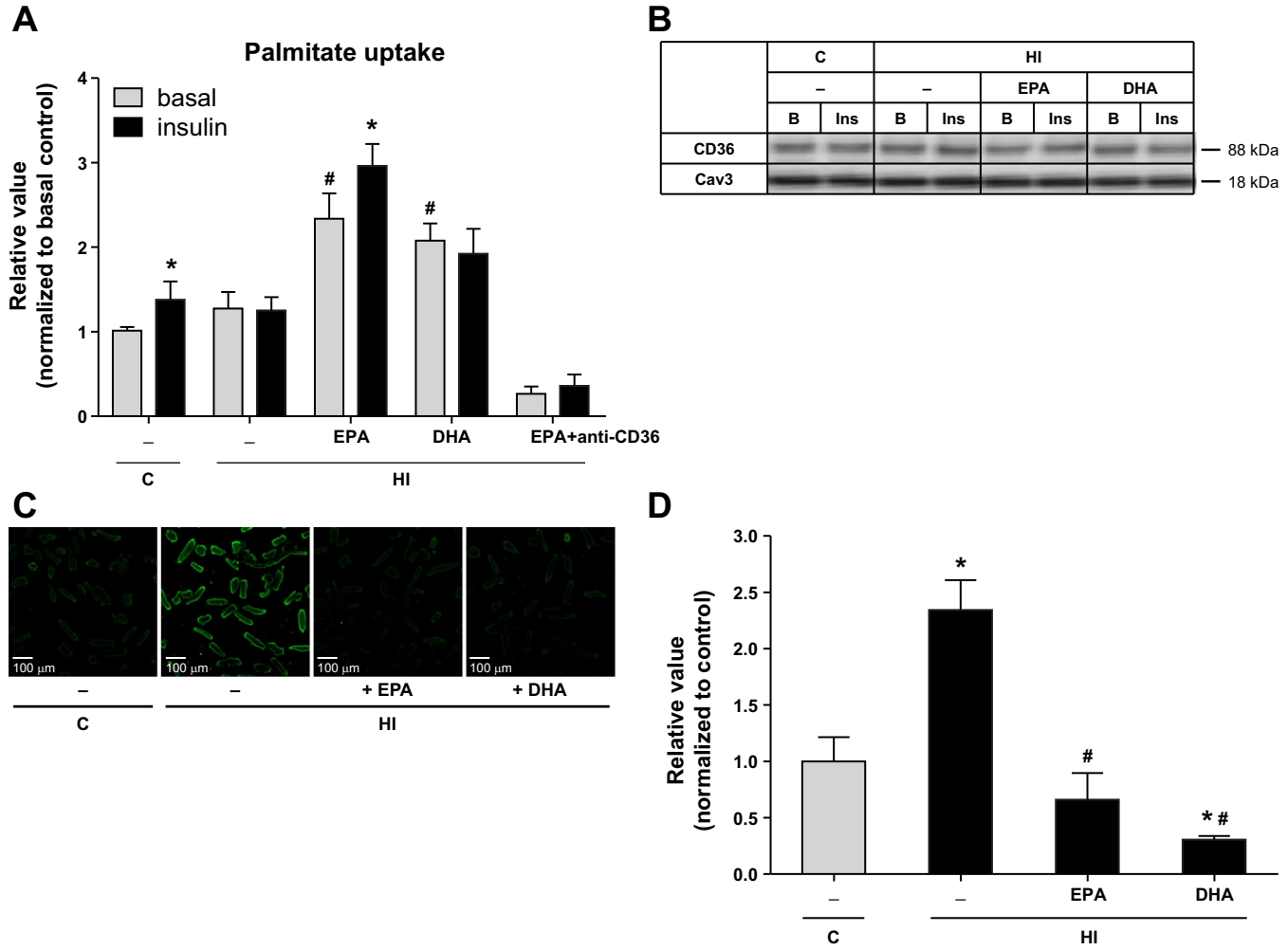


Fig. 4. Effect of ω-3 PUFAs on palmitate uptake, total expression level, and cell-surface content of CD36. Cardiomyocytes were pretreated and stimulated with insulin as described in the legend of Fig. 1. For measurement of palmitate uptake [^{14}C]palmitate was added to medium for last 10 min of incubation. *A*: palmitate uptake data obtained from cardiomyocytes cultured in control (C) or high insulin (HI) medium in the absence or presence of ω-3 PUFAs during basal and insulin-stimulated conditions. Also shown are data from cardiomyocytes cultured in the presence of EPA coincubated with anti-CD36 antibody. Results are represented as mean values ± SE ($n = 11$ for treatment with EPA, $n = 4$ for treatment with DHA). *$P < 0.05$ vs. corresponding basal value; #$P < 0.05$ vs. basal value in the absence of ω-3 PUFAs. *B*: representative Western blot analyses ($n = 4$) of total CD36 in cardiomyocytes cultured in control or HI medium in the absence or presence of ω-3 PUFAs during basal (B) and insulin-stimulated conditions (Ins). Caveolin 3 (Cav3) was used as loading control. *C*: representative confocal images ($n = 3$) showing surface staining of CD36 in cardiomyocytes cultured in control or HI medium in the absence or presence of 200 μM EPA or DHA. *D*: quantification of the signals from confocal images. Results are represented as mean values ± SE. *$P < 0.05$ vs. control value; #$P < 0.05$ vs. HI value in the absence of ω-3 PUFAs.

PUFAs. Hence, the ω-3 PUFAs-mediated increase in substrate uptake remains unresolved.

The presently observed ability of ω-3 PUFAs to antagonize the development of insulin resistance in cardiomyocytes at the level of the Akt/PKB pathway or glucose uptake is in line with previous results in other cellular models. Thus improved insulin-stimulated glucose uptake was observed after the treatment with EPA or DHA in skeletal muscle cells, as well as in adipocytes cocultured with macrophages (1, 33).

Insulin-stimulated glucose uptake in cardiac myocytes is mediated by translocation of the glucose transporter GLUT4 from intracellular compartments to the plasma membrane (14). In accordance with previous studies (1, 25) we found that the preserved insulin sensitivity in cardiomyocytes coincubated with EPA or DHA during HI conditions was not associated with changes in the total cellular content of GLUT4. Because of experimental limitations, we were not able to measure GLUT4 translocation (surface GLUT4). However, several reports have shown that insulin-stimulated glucose transport in the heart appears to be primarily mediated by the Akt/PKB signaling pathway, and that defects in GLUT4 translocation are associated with alterations in this pathway (7, 9, 20). Furthermore, the Rab GTPase-activating protein AS160 is a substrate for Akt/PKB and serves as a link between insulin activation of Akt/PKB and the subsequent translocation of GLUT4 to the cell surface (41). As expected, induction of insulin resistance in cardiomyocytes (in response to long-term HI exposure) was associated with reduced phosphorylation of both Akt/PKB and AS160.

One should bear in mind, however, that Akt/PKB is not the only kinase responsible for phosphorylation of AS160. For example, AMPK, independently of insulin, is also able to

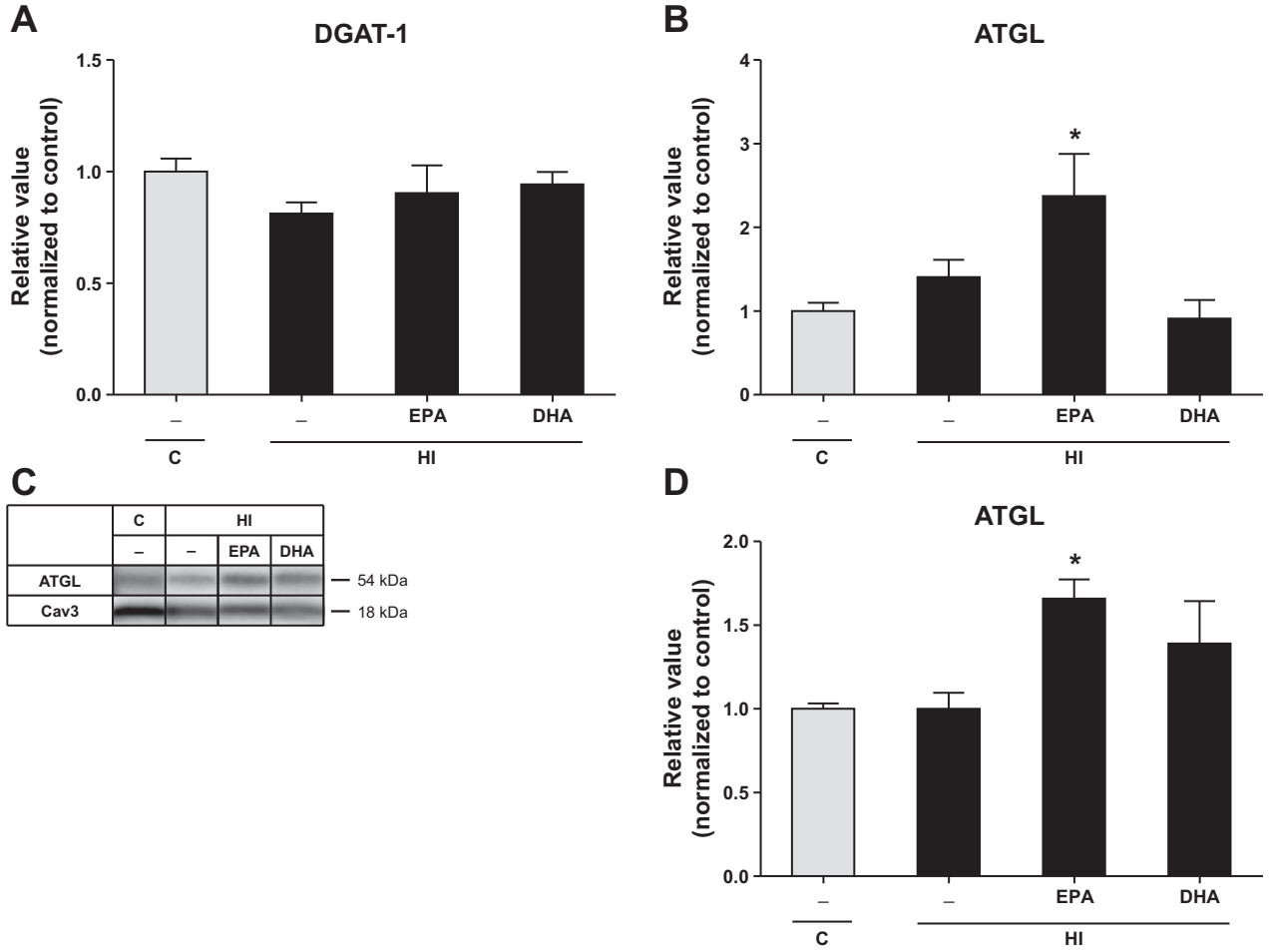


Fig. 5. Effect of ω-3 PUFAs on fatty acid-metabolic enzymes. Cardiomyocytes were pretreated as described in the legend of Fig. 1. *A* and *B*: mRNA expression of acyl-CoA:diacylglycerol acyltransferase-1 (DGAT-1) and adipose triglyceride lipase (ATGL) in cardiomyocytes cultured in control (C) or high insulin (HI) medium in the absence or presence of ω-3 PUFAs. Levels of DGAT-1 and ATGL were quantified relative to the housekeeping control gene GAPDH. *C*: representative Western blot analyses ($n = 4$) of ATGL in cardiomyocytes cultured in control (C) or high insulin (HI) medium in the absence or presence of ω-3 PUFAs. Caveolin 3 (Cav3) was used as loading control. *D*: quantification of the signals from Western blot analyses corrected for loading control. Results are represented as mean values ± SE ($n = 4$). *$P < 0.05$ vs. basal value in the absence of ω-3 PUFAs.

phosphorylate AS160 (24) and stimulate glucose uptake in the heart (6, 40). Nonetheless, our present results indicate that EPA or DHA do not activate AMPK. However, still other mechanisms could be responsible for preservation of insulin signaling and insulin-stimulated glucose uptake by ω-3 PUFAs (especially EPA) during culturing of cardiomyocytes under insulin resistance-inducing conditions.

Interestingly, while EPA treatment preserved both insulin-induced Akt/PKB phosphorylation and insulin-stimulated glucose uptake, DHA improved only Akt/PKB phosphorylation. This should not be entirely surprising, however, as some studies have already shown that the PKB/Akt pathway and glucose uptake downstream of insulin might be disconnected (17, 50, 52). The subcellular trafficking machinery dedicated to GLUT4 translocation includes, however, activation of proteins in addition to Akt/PKB (e.g., SNARE proteins, motor proteins, coat proteins, etc.), and the apparent discrepancy between EPA and DHA on GLUT4 translocation could therefore be explained by a lower efficacy of DHA to interact with these other proteins.

In contrast to the preservation of the insulin effect on phosphorylation of Akt/PKB in cardiomyocytes cultured under HI conditions, insulin signaling at the level of mTOR appeared not to be preserved. It should be remembered that activation of mTOR is a very complex process involving Akt/PKB, but also numerous other kinases and regulatory proteins, and it is under control of several feedback loops as well (49). Thus it seems that EPA and DHA are able to selectively protect Akt/PKB, but not other proteins needed for mTOR activation.

Redistribution of CD36 from intracellular stores to the plasma membrane and lipid accumulation are hallmark changes occurring in the heart during diet-induced obesity and insulin resistance (5, 28). Thus cardiomyocytes which were made insulin resistant by HI displayed elevated sarcolemmal CD36 content. EPA and DHA increased palmitate uptake, but, surprisingly, this response was accompanied neither by changes in the total expression of CD36 nor by translocation of CD36 to the plasma membrane. On the contrary, inclusion of EPA or DHA significantly prevented the robust translocation of CD36 to the sarcolemma, which was otherwise seen in the insulin-resistant cardiomyocytes. At a first glance, this observation seems paradoxical, but on the other hand it shows that exposure to EPA or DHA prevented one of the main processes

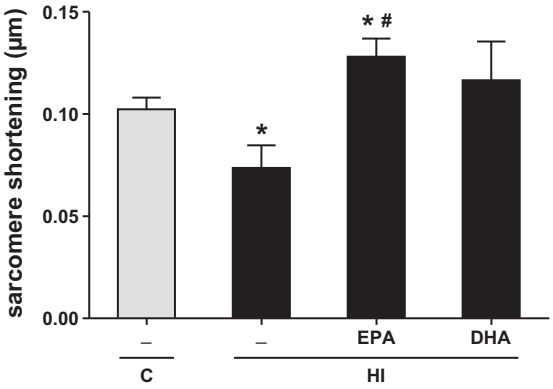


Fig. 6. Effect of ω-3 PUFAs on peak sarcomere shortening. Cardiomyocytes were preincubated for 48 h in control medium (C) or in medium supplied with 100 nM insulin (HI) in the absence or presence of 200 μM EPA or DHA. Subsequently peak sarcomere shortening was recorded under 1 Hz and 5 ms of electrical pulsated cardiomyocytes. Results are represented as mean values ± SE ($n = 6$). *$P < 0.05$ vs. control value; #$P < 0.05$ vs. value in the absence of ω-3 PUFAs.

involved in the development of insulin resistance, namely persistent CD36 relocation to the sarcolemma. Our results are also consistent with results by Madonna and colleagues (30), who observed a decreased plasma membrane content of CD36 after treatment with EPA or DHA in human microvascular endothelial cells.

The fact that the EPA- or DHA-induced elevation of palmitate uptake was not paralleled by elevated presence of CD36 at the sarcolemma could indicate that EPA and DHA open alternative avenues for palmitate uptake, which bypass the CD36 transporter. One possibility could be that the presence of EPA or DHA alters the fluidity of the sarcolemma by incorporation into the phospholipid bilayer, allowing for increased uptake of palmitate through passive diffusion (19). Apparently, this was not the case, since blocking of CD36 during EPA exposure by specific anti-CD36 antibody almost totally prevented the palmitate uptake. Perhaps, in addition to their prevention of CD36 permanent relocation to sarcolemma, both EPA and DHA alter the bilayer organization around CD36, thereby increasing the LCFA transport function of this membrane protein.

Besides at the level of CD36, this study indicates that EPA is also able to influence cardiomyocyte fatty acid handling at the level of lipid breakdown via upregulation of ATGL. It has previously been shown that HI cultured cardiomyocytes display a twofold accumulation of intracellular triacylglycerol (5). Here we show that ATGL expression was not altered upon HI treatment. This might suggest that myocellular lipid accumulation in HI conditions is due to permanent CD36 translocation and increased fatty acid uptake, but not to a reduction in triacylglycerol hydrolysis. Yet, the ability of EPA to upregulate ATGL could contribute to the beneficial effect of this fatty acid to preserve insulin signaling in cardiomyocytes during conditions of lipid overload, i.e., via redirecting fatty acids from triacylglycerol storage towards mitochondrial β-oxidation. ATGL upregulation would downregulate not only triacylglycerol, but also diacylglycerol and ceramide levels, and thereby relieve the blockade of insulin signaling in cardiomyocytes during lipid overexposure.

Finally, impaired contractile function has been reported in isolated cardiomyocytes in insulin-resistant states, probably due to alterations in transsarcolemmal ion transport (38). The present observation that EPA (and probably DHA) improves contractile function of cardiomyocytes cultured under HI conditions is a novel finding, which should be explained in terms of the concomitantly improved insulin sensitivity, although determination of the cellular and molecular mechanisms involved requires further investigations.

Concluding remarks. In summary, the present study shows that EPA and DHA counteract the development of insulin resistance in isolated cardiomyocytes incubated under insulin resistance-evoking conditions by preserving the phosphorylation state of key proteins in the insulin signaling cascade and by preventing persistent relocation of CD36 to the sarcolemma. To our knowledge, this is the first study extending the beneficial effects of ω-3 PUFAs to preservation of contractile function of the heart at the cellular level and in the context of insulin resistance.

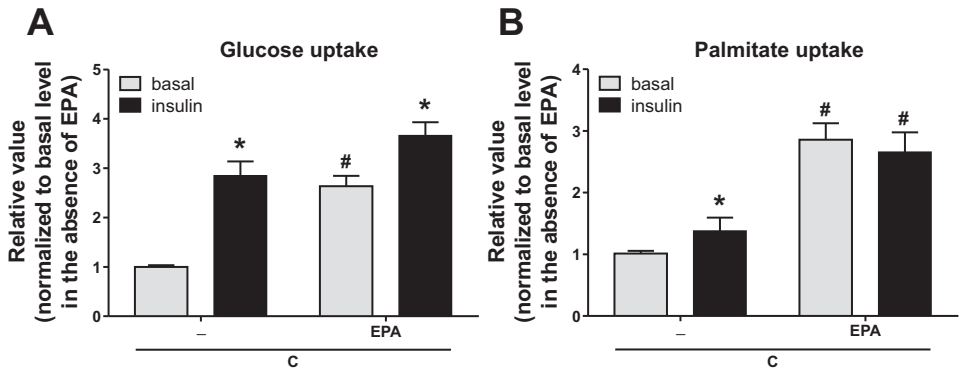


Fig. 7. Effect of EPA on substrate uptake in insulin-sensitive cardiomyocytes. Cardiomyocytes were preincubated for 48 h in control medium (C) in the absence or presence of 200 μM EPA. Thereafter, cells were washed and allowed to recover for 30 min prior to short-term (25 min) incubation with or without insulin (100 nM). For measurement of substrate uptake [^{3}H]deoxyglucose and [^{14}C]palmitate were added to medium for the last 10 min of incubation. Glucose (*A*) and palmitate (*B*) uptake data obtained from cardiomyocytes cultured in control medium in the absence or presence of EPA during basal and insulin-stimulated conditions. Results are represented as mean values ± SE ($n = 4$ for glucose uptake; for palmitate uptake $n = 14$ for nonexposed cells and $n = 4$ for EPA-exposed cells). *$P < 0.05$ vs. basal value; #$P < 0.05$ vs. corresponding values in the absence of EPA.

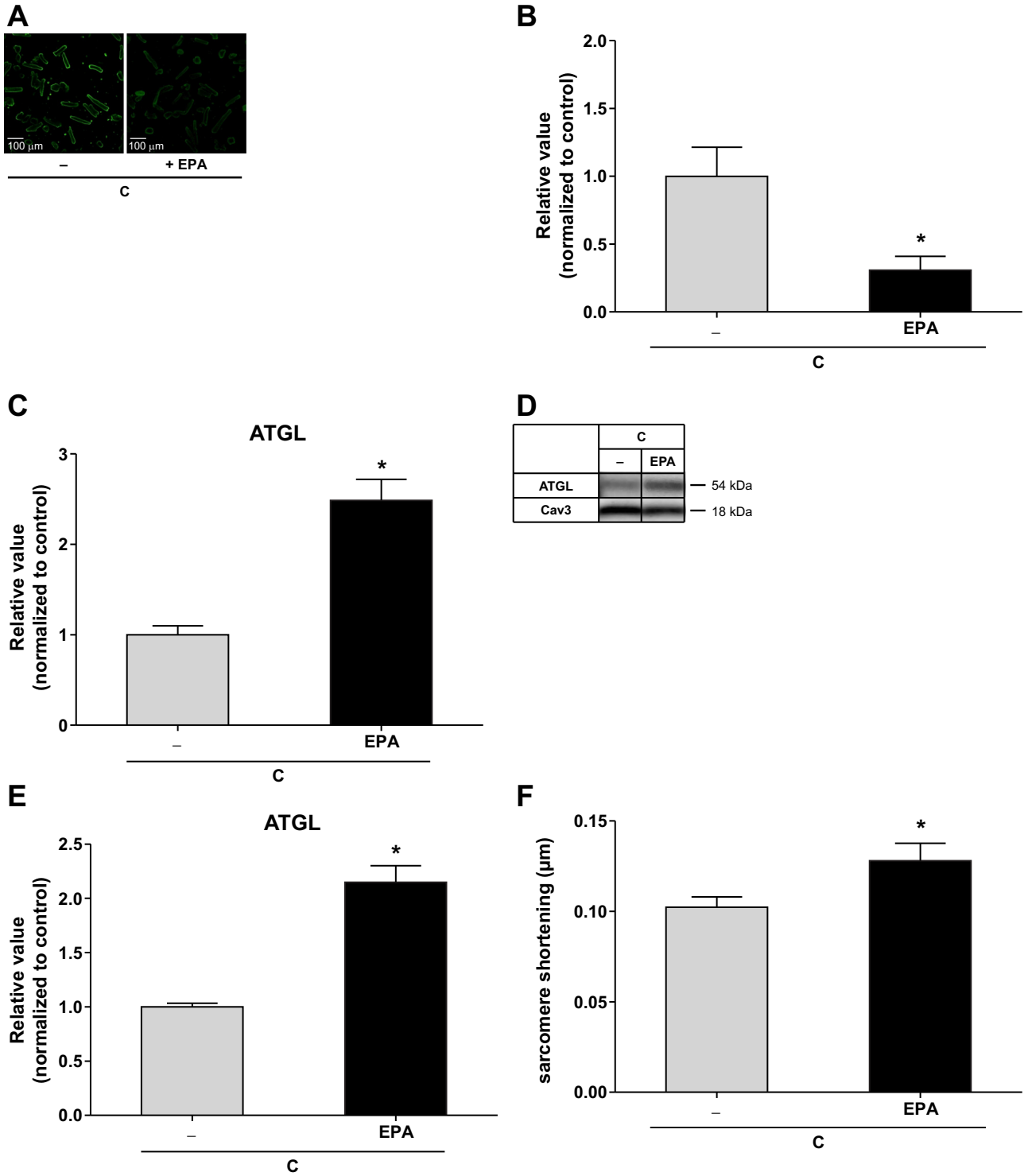


Fig. 8. Effect of EPA on cell-surface content of CD36, expression of ATGL, and peak sarcomere shortening in insulin-sensitive cardiomyocytes. Cardiomyocytes were preincubated for 48 h in control medium (C) in the absence or presence of 200 μM EPA. *A*: representative confocal images showing surface staining of CD36. *B*: quantification of the signals from confocal images. *C*: mRNA expression of ATGL. Level of ATGL was quantified relative to the housekeeping control gene GAPDH. *D*: representative Western blot analyses of ATGL. Caveolin 3 (Cav3) was used as loading control. *E*: quantification of the signals from Western blot analyses corrected for loading control. *F*: peak sarcomere shortening data obtained from cardiomyocytes cultured for 48 h in control medium in the absence or presence of EPA. Results are represented as mean values ± SE ($n = 3–6$). *$P < 0.05$ vs. value in the absence of EPA.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.F., J.F.G., J.J.L., and T.S.L. conception and design of research; V.F., Y.A., N.T.H., and W.A.C. performed experiments; V.F., Y.A., and J.J.L. analyzed data; V.F., Y.A., J.J.L., and T.S.L. interpreted results of experiments; V.F. prepared figures; V.F. and T.S.L. drafted manuscript; V.F., Y.A., J.F.G., J.J.L., and T.S.L. edited and revised manuscript; V.F., Y.A., N.T.H., W.A.C., P.J.S., J.F.G., J.J.L., and T.S.L. approved final version of manuscript.

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