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Linoleic Acid Permeabilizes Gastric Epithelial Cells by Increasing Connexin43 Levels in the Cell Membrane Via a GPR40- and Akt-Dependent Mechanism

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Abstract

Linoleic acid (LA) is known to activate G-protein coupled receptors and connexin hemichannels (Cx HCs) but possible interlinks between these two responses remain unexplored. Here, we evaluated the mechanism of action of LA on the membrane permeability mediated by Cx HCs in MKN28 cells. These cells were found to express connexins, GPR40, GPR120, and CD36 receptors. The Cx HC activity of these cells increased after 5 min of treatment with LA or GW9508, an agonist of GPR40/GPR120; or exposure to extracellular divalent cation-free solution (DCFS), known to increase the open probability of Cx HCs, yields an immediate increase in Cx HC of similar intensity and additive with LA-induced change. Treatment with a CD36 blocker or transfection with siRNA-GPR120 maintain the LA-induced Cx HC activity. However, cells transfected with siRNA-GPR40 did not show LA-induced Cx HC activity but activity was increased upon exposure to DCFS, confirming the presence of activatable Cx HCs in the cell membrane. Treatment with AKTi (Akt inhibitor) abrogated the LA-induced Cx HC activity. In HeLa cells transfected with Cx43 (HeLa-Cx43), LA induced phosphorylation of surface Cx43 at serine 373 (S373), site for Akt phosphorylation. HeLa-Cx43 but not HeLa-Cx43 cells with a S373A mutation showed a LA-induced Cx HC activity directly related to an increase in cell surface Cx43 levels. Thus, the increase in membrane permeability induced by LA is mediated by

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an intracellular signaling pathway activated by GPR40 that leads to an increase in membrane levels of Cx43 phosphorylated at serine 373 via Akt.

Keywords

polyunsaturated fatty acid (PUFA); FFAR1; connexon (hemichannel); transmembrane permeability; protein phosphorylation; Akt kinase; G protein-coupled receptor (GPCR)

1. INTRODUCTION

Connexin (Cx) proteins are encoded by a gene family composed by 21 and 20 members in human and mouse, respectively [1, 2], and they are assembled into hexamers to form hemichannels (HCs) or connexons, and dodecamers to form gap junction channels (GJCs) connecting two cells. HCs can act as relatively non-selective channels and are found at the cell surface of most vertebrate cells [1, 3-5]. HCs are often involved in paracrine and autocrine cellular signaling, being membrane pathways for releasing signaling molecules (e.g., ATP, NAD⁺ and glutamate) to the extracellular space [3]. Different tissues express different Cxs depending on their developmental and physiologic state, and they are named alphanumerically with the prefix Cx followed by the molecular mass of the human family member in kilodaltons (e.g., Cx43) [1, 6].

Experimental evidence suggests that functional HCs and GJCs are involved in physiological and pathophysiological cell responses. In the gastrointestinal system, they might participate as paracellular permeability pathway in intestinal epithelial cells [7] or intestinal innate immune defense [8]. Moreover, in tanocytes and skeletal muscle, Cx43 HCs or pannexin 1 channels (Panx1 Chs), respectively, allow diffusional entry of molecules such as glucose [9, 10], so they could be thought of as membrane pathways for cellular uptake of nutrients. At least 7 different Cx variants have been described in the human gastrointestinal system [11], with the three Cxs found in gastric cells being connexin 26 (Cx26), Cx32 and Cx43 [12]. The functional roles of these Cxs are poorly understood but one proposed by Guttman et al. [13], postulated that Cx43 HCs present in intestine are involved in transport of water, at least under pathological conditions such as in diarrhea caused by bacterial infections. Also, the expression of Cx26 is associated with intestinal type-gastric cancer and Cx32 and Cx43 with *Helicobacter pylori*-associated gastric tumorigenesis [14, 15].

In the gastrointestinal tract, free fatty acids (FFAs) are essential nutrients but they also play important roles as signaling molecules [17, 18] in various physiological processes [19, 20]. However, knowledge about the regulation of HCs (or GJCs) by FFAs in the epithelium of the gastrointestinal tract is very limited and is incompletely understood in other cell types [21-24].

FFAs exert at least some of their biological signaling effect through the G-protein coupled receptor (GPCR) family [25, 26] of which at least 5 genes (GPR40, GPR41, GPR43, GPR84 and GPR120) and one pseudo gene (GPR42) have been described [27]. These receptors have different affinities for different FFAs with those having medium and long aliphatic chains being the best agonists for GPR40 (or FFAR1) and/or GPR120 [27, 28]. In addition, CD36

(a ubiquitous membrane glycoprotein) has been shown to mediate responses to FFAs, by acting as a lipid sensor [29, 30]. These three types of receptors (i.e. GPR40, GPR120 and CD36) are activated by long chain fatty acids [27, 28, 31]. Probably the two most well studied FFAs are linoleic and α -linolenic acids, both of which are precursors of the longer aliphatic chains ω -6 and ω -3 fatty acids, respectively, and are essential nutrients in the human diet [32-35].

Linoleic acid (LA) has been shown to modulate the functional state of Cx46 HCs [21]. Moreover, in HeLa cells transfected with different Cxs, our group reported that LA increases Cx26, Cx43 and Cx45 HC activity, and this effect is associated with elevated intracellular free Ca^{2+} concentration and activation of the PI3K/Akt-dependent pathway [24]. However, the molecular mechanisms involved in this response remain unknown and as does the generality of the response of cell types that have actual contact with FFAs. Hence, we evaluated the mechanism of action of LA on the functional state of Cx43 HCs in cells derived from human gastric epithelium (MKN28 cells). To further understand the mechanism of action, we also used HeLa cells transfected with Cx43 or Cx43 lacking an Akt phosphorylation site. We found that LA induces an increase in Cx43 HC activity by increasing the number of hemichannels in the cell membrane through an extracellular Ca^{2+} independent mechanism that sequentially involves activation of GPR40 and Akt followed by phosphorylation of Cx43 at serine 373.

2. MATERIAL AND METHODS

2.1. Reagents

Linoleic acid (LA), GW9508, ethidium (Etd^+) bromide and lanthanum (La^{3+}) chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA); Akt inhibitor VIII (AKTi) from Calbiochem (Merck KGaA, Darmstadt, Germany); GPR40 and Negative control siRNA (Neg siRNA) were obtained from Qiagen (Valencia, CA, USA), polyclonal antibodies anti-GPR40 and GPR120 from Abcam (Cambridge, MA, USA). A custom made previously described anti-pS373 Cx43 antibody was used [36]. Monoclonal antibody anti- α -tubulin was obtained from Sigma-Aldrich, and anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

2.2. Cell culture

HeLa-Parental cells were obtained from ATCC (CCL-2; ATCC, Rockville, MD) and HeLa cells stably transfected with rat Cx26 cDNA were kindly provided by Dr. Bruce Nicholson, Department of Biochemistry at the University of San Antonio, USA. HeLa cells stably transfected with mouse Cx32 or Cx43 cDNA were kindly provided by Dr. Klaus Willecke from the Limus Institute, Bonn University, Germany. MKN28 cells (cell line derived from human gastric epithelium) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa cells were cultured in DMEM-10% fetal bovine serum (GIBCO) with 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. Puromycin (10 $\mu\text{g}/\text{ml}$) or Geneticin (250 $\mu\text{g}/\text{ml}$) were used to select transfected cells. Untransfected HeLa-Parental cells were used as controls. MKN28 cells were cultured in RPMI-10% fetal bovine serum

with 100 U/ml penicillin, 100 µg/ml streptomycin sulfate. Cells were used 36 – 48 h after seeded.

2.3. RT-PCR assay

Total RNA was isolated from MKN28 or HeLa cells using Trizol® Reagent following the manufacturer instructions (Ambion, USA). Aliquots of 2 µg of total RNA were reversed transcribed into cDNA using MMLV-reverse transcriptase (Fermentas, USA) and mRNA levels were evaluated by PCR amplification (GoTaq® Flexi DNA Polymerase; Promega, USA). The primers used were GAPDH sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCCTGTTGCTGTA-3'; GPR40 sense 5'-TGGCCCACTTCTTCCCACTC-3', antisense 5'-CAGGAGAGAGAGGCTGAAGC-3', GPR120 sense 5'-TTGCACACTGATTTGGCCCA-3', antisense 5'-CTTCCACTCATTCCTGCACA-3'; Cx32 sense 5'-CAGCTCATCTAGTTTCCACC-3', 5'-ACCACCTCGGCCACATTGA-3'; and Cx43 sense 5'-GTCCCTGGCCTTGAATATCA-3', antisense 5'-TCTGGTTATCATCGGGGAAA-3'. For Cx26 was previously described [14]. The expected size products were: GPR40, 317 bp; GPR120, 463 bp; Cx43, 371 bp; Cx32 392 bp and GAPDH: 452 bp.

2.4. Western blot assays

Cells were washed twice with ice-cold PBS (pH 7.4), harvested by scraping with a rubber policeman in 80 µl of solution containing protease and phosphatase inhibitor cocktail (Thermo Scientific, Pierce, Rockford, IL, USA), placed on ice and then sonicated (Ultrasonic cell disrupter, Microson, Ultrasons, Annemasse, France). Blots were incubated overnight with polyclonal rabbit immunopurified anti-GPR40, GPR120, Cx26 or Cx43 antibodies diluted with 5% non-fat milk in TBS. Then, they were rinsed with TBS and incubated for 1 h at room temperature with horse radish peroxidase-conjugated goat anti-rabbit IgG antibodies at the appropriate dilution in PBS with 5% non-fat milk. After repeated rinses, immunoreactive proteins were detected using ECL reagents (Pierce biotechnology, Rockford, IL) according to the manufacturer's instructions.

2.5 Indirect immunofluorescence analysis

Cells grown on glass coverslips were washed three times with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 5 min at room temperature. Cells were then washed three times, and permeabilized and blocked in PBS containing 1% BSA and 0.25% Triton X-100. Cells were incubated for 1 hour at room temperature with primary anti-GPR40 or anti-GPR120 antibodies previously dissolved in the washing solution containing 0.2% BSA in PBS and 0.025% Triton X-100. Cells were then washed three times to remove the unbound antibody. Afterwards, cells were incubated for 1 hour at room temperature with anti-rabbit secondary antibody labeled with Cy2 (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA), then washed three times and mounted with DABCO mounting medium (Gelvatol, Sigma Aldrich, St. Louis, MO, USA) containing 1:2,000 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Aldrich, St. Louis, MO, USA) and imaged using the immunofluorescence microscopy. Secondary antibodies alone were used as negative controls.

2.6. Dye uptake

The HC activity was measured using a published dye uptake method [9]. In brief, the cells were plated onto glass coverslips and after 36–48 h they were washed twice with Locke's buffer (in mM: 154 NaCl, 5.4 KCl, 2.3 CaCl₂, 1.5 MgCl₂, 5 glucose, 5 Hepes, pH 7.4) containing 5 μM ethidium (Etd⁺), a membrane-impermeant cationic dye, and images were recorded (at regions of interest in different cells) every 30 s during 30 min using a Nikon Eclipse Ti inverted microscope (Japan) and NIS-Elements software. Basal fluorescence signal was recorded (during 5 min) in cells only in the presence of Locke's buffer that contained divalent cations. Then, LA was added to the same buffer and recorded for ~15 min, and at the end of the experiment La³⁺ was added. Image analysis was performed with ImageJ 1.46r software (NIH, USA).

2.7. siRNA Transfection

MKN28 and HeLa cells were transfected with GPR40 or control (Neg) siRNAs (Qiagen) at a final concentration of 100 nM using Lipofectamine LTX and PLUS Reagents as described in the manufacturer's instructions (Invitrogen) for the 35 mm dish format. pEGFP-N1 vector was included to determine transfection efficiency. After 36–48 h of transfection, cells were used for Etd⁺ uptake, Ca²⁺ signal evaluation and immunofluorescence experiments.

2.8. Levels of Cx43 HCs at the cell surface

Cell surface proteins were biotinylated as described previously [37]. In brief, cell cultures were grown in 100 mm-dishes, washed 3 times with ice-cold Hank's saline solution pH 7.4 and incubated (30 min, at 4°C) in 3 ml of 0.5 mg/ml sulfo-NHS-SSbiotin (Pierce, Rockford, IL). The cells were washed 3 times with a saline solution containing 15 mM glycine pH 8.0 to neutralize unreacted biotin and finally harvested in saline solution with a cocktail of protease and phosphatase inhibitors. Then cells were sonicated on ice and protein concentration was determined. Immobilized NeutrAvidin (Pierce) was added (1 ml of NeutrAvidin per 3 g of biotinylated protein) and the mixture was incubated for 1 h at 4°C. Then 1 ml of washing buffer (saline solution, pH 7.4, 0.1% SDS and 1% NP-40) was added, the mix was centrifuged (2 min, 14,000 rpm, 4°C), and the supernatant was discarded. After performing this procedure 3 times, 40 μl Hanks solution buffer at pH 2.8 containing 0.1 M glycine was added to the pellet to release the proteins bound to biotin. The pellet was resuspended and centrifuged. The supernatant was transferred to a new 1.5 ml Eppendorf tube, and the pH was adjusted immediately by adding 10 μl of 1 M Tris pH 7.4. Finally, relative levels of protein were evaluated by Western blot.

2.9. Data Analysis

For each group of data, results were expressed as mean ± SEM, and n represents the number of independent experiments or the number of cells analyzed as indicated. For Etd⁺ uptake experiments, each mean corresponds to the average of at least 20 cells. Data sets were compared by one-way analysis of variance (ANOVA) followed by a Bonferroni's post-test. Differences were considered significant at p 0.05. The analyses were performed with GraphPad Prism 5 software for Windows (1992–2007, GraphPad Software).

3. RESULTS

3.1 Human gastric epithelial cells present Cx HC activity

Connexin expression has been previously demonstrated in different sections of the gastrointestinal tract [6, 7, 11]. Here we show that the MKN28 cell line, derived from human gastric epithelium [38], expresses the mRNA for Cx26, Cx32 and Cx43 (Figure 1A). Since these Cxs can form hemichannels (HCs) in cell membrane [1], we tested to see if MKN28 cells express functional Cx HCs. Under basal conditions, most Cx HCs remain in the closed state (i.e., show low open probability). However, their presence can be demonstrated by increasing their open probability, for example, by drastically reducing the extracellular concentration of divalent cations [39, 40]. To this end, cells were bathed with divalent cation-free solution (DCFS), which has been shown to promote HC opening [41-43]. The functional state of Cx HCs was monitored by measuring the Etd⁺ uptake over time (Fig. 1B, C). Etd⁺ is a membrane-impermeant cationic dye that becomes fluorescent when intercalated in nucleic acid strands [44]. A rapid increase in fluorescence intensity was observed upon DCFS application that was drastically reduced by lanthanum ions (La³⁺, 200 μM) (Figure 1C, D), a known Cx HC blocker [42, 43]. The quantification of Etd⁺ uptake rate revealed a ~3-fold increase in DCFS, and this response was blocked by La³⁺ to values even lower than that recorded under control conditions (Figure 1D). These results indicate there is likely a low level of Cx HC activity even under basal conditions.

3.2. Linoleic acid (LA) increases the Cx HC activity through a mechanism independent of extracellular divalent cations

Since MKN28 cells were found to express Cx HCs, we then tested if the activity of these hemichannels is affected by linoleic acid (LA). Approximately 5 min after the application of 100 μM LA, Etd⁺ uptake increased several-fold and this response was completely blocked by La³⁺ (Figure 2). Notably, the intensity of this effect was similar to that induced by DCFS though the DCFS-induced rise did not show the initial 5 min lag in uptake, suggesting different mechanisms of activation for these treatments. To further study this possibility, HeLa-Cx43 cells were first exposed to DCFS and 10 min later treated with 100 μM LA for 10 min (Figure 2C) or vice versa (Figure 2D). In both cases, after 20 min, 200 μM La³⁺ was applied (Figure 2C and D). The LA concentration used is known to induce its maximal effect on Cx HC [24] and complete replacement of the media with DCFS should yield the maximal open probability of Cx HCs via this mechanism. Since DCFS treatment after LA application, and vice versa show further increases in HC activity, it seems likely that these two treatments increase hemichannel activity via independent mechanisms.

3.3. The LA-induced Cx HC activity requires G-protein receptor 40 (GPR40)

Diverse effects of different FFAs have been linked to G-protein coupled receptors (GPCRs) [25, 26]. In particular, the effects of medium and long chain FFAs were found associated with two different receptors: GPR40/ FFAR1 (Free Fatty Acids Receptor 1) and GPR120 [26, 45]. Also, another possible candidate for FFA receptor is the broadly expressed membrane glycoprotein CD36 [46]. Although the effect of LA, a ω-6 long chain FFA, has been associated with GPR40 [25, 45] to our knowledge the membrane transduction of FFA in MKN28 cells has not been described. We found that MKN28 cells express the mRNA for

GPR40, GPR120 mRNA (Figure 3A), and CD36 (Supp Figure 1A). Moreover, the application of GW9508 (40 μ M), an agonist of GPR40 and GPR120 [19, 47], increased the Etd⁺ uptake after a delay of about 5 min (Figure 3B). The increase in Etd⁺ uptake rate was ~3-fold and the application of La³⁺ reduced the Etd⁺ uptake to values lower than that of control cells (Figure 3C). The possible participation of CD36 in the LA-induced Cx HC activity in MKN28 cells was discarded because treatment with 100 μ M sulfosuccinimidyl oleate, a CD36 inhibitor, did not affect the LA-induced Etd⁺ uptake of MKN28 cells (Supp Figure 1B, 1C).

To further confirm the involvement of GPR40 in the LA-induced increase in membrane permeability, MKN28 cells were transfected with a siRNA for GPR40. The knock-down of GPR40 was confirmed by RT-PCR and immunofluorescence analyses, respectively, but use of a control siRNA (Neg siRNA) did not (Figure 4A). In MKN28 cells transfected with GPR40-siRNA, LA (100 μ M) did not affect Etd⁺ uptake, but cells transfected with Neg-siRNA showed an evident increase in Etd⁺ uptake and this response was blocked with La³⁺ (200 μ M) (Figure 4B, 4Ca). Nevertheless, we did not know if the absence of response to LA found in cells transfected with siRNA-GPR40 was due to knock-down of GPR40 or down regulation of Cx HC activity induced by the GPR40 knock-down procedure. To test for reduced Cx HCs, cells were transfected cells with Neg- or GPR40- siRNA and treated with DCFS. Both preparations showed similar increases in dye uptake and inhibition by La³⁺ (Figure 4Cb), indicating that the amount of Cx HCs found in the cell surface was unaffected by the procedure and thus, the lack of response was due to the absence of effective GPR40-mediated signal transduction. Moreover, the effect of LA over Cx HC activity in MKN28 cells apparently is independent of GPR120 activity, because cells transfected with siRNA-GPR120 showed drastic reduction in GPR120 reactivity and levels of GPR120 mRNA but presented similar increase in Etd⁺ uptake induced by 100 μ M LA (Supp Figure 2A y 2B).

Previously, a report found that LA increases the hemichannel activity in HeLa cells transfected with Cx26, Cx43 or Cx45 [24], but the possible involvement of GPR40 remained unknown. Here, we show that HeLa cells transfected with Cx26, Cx32, Cx43 or Cx45 express GPR40 (Figure 5A) and the LA-induced dye uptake was reduced in cells transiently transfected with siRNA-GPR40 (Figure 5B and C). Similarly, the increase in dye uptake rate of HeLa cells transfected with Cx26 or Cx32 or Cx43 was completely abrogated by transfection with siRNA-GPR40 (Figure 5C). In all transfectant cells, Etd⁺ uptake was inhibited by La³⁺ (Figure 5B and C).

3.4. The LA-induced increase in membrane permeability requires a Cx43 Akt phosphorylation site

The LA-induced increase in Cx HC activity has been shown to involve the activation of Akt [24]. Hence, we wanted to determine the involvement of Akt in the Cx HC membrane permeability of MKN28 cells treated with LA. We found that LA-induced dye uptake was completely abrogated in MKN28 cells preincubated for 30 min with 10 μ M AKTi, an Akt kinase inhibitor [37] (Figure 6A). Quantification of the Etd⁺ uptake rate revealed that AKTi treatment apparently lead to inhibition below control values even in cells not treated with LA (Figure 6B), suggesting basal Cx HC activity depends on basal Akt activity and possibly

phosphorylation of Cx43. Cx43 have been shown to be phosphorylated by Akt primarily at serine 373 [36, 48, 49]. To study the possible participation of Cx43 phosphorylation via Akt, we studied the effect of LA on Cx HCs in HeLa-Cx43-S373A cells. As shown above, HeLa cells transfected with wild type Cx43 (Cx43-WT) showed an evident LA-induced increase in Etd⁺ uptake rate (Figure 5C). However, dye uptake of HeLa cells transfected with Cx43-S373A was not modified by LA (Figure 6C). HeLa-Cx43-S373A cells bathed with DCFS showed rapid activation of Cx HCs that was blocked with La³⁺ (200 μM). Thus, the effect of Cx43 mutation at serine 373 appears to be specific to the LA-induced pathway as the mutation modifies neither the amount of available Cx HC in the cell membrane nor their sensitivity to low concentration of divalent cations.

The increase in Cx HC activity induced by LA could result from an increase open probability of HCs already present in the cell membrane and/or from an increase in amount of available Cx HCs in the cell surface. Here, we tested the latter possibility using biotinylation of cell surface membrane proteins followed by precipitation and Western blot analysis of Cx43. Cx43 was readily detected on the surface of the HeLa-Cx43 cells (Figure 7A). The levels of cell surface Cx43 in cells under control conditions was not significantly affected by 30 min incubation with 10 μM AKTi. Moreover, the application of 100 μM LA for 15 min approximately doubled the amount of cell surface Cx43 and that increase was abrogated in cells preincubated with 10 μM AKTi for 30 min (Figure 7). Also, phosphorylation of Cx43 at S373 occurred only after application of LA and this response was completely prevented by AKTi (Figure 7A).

4. DISCUSSION

Here, we show that linoleic acid (LA), an essential unsaturated fatty acid, increases the permeability of the plasma membrane by increasing the activity of Cx HCs in gastric epithelial cells through a mechanism that involves a membrane receptor (GPR40) and activation of an Akt-dependent intracellular signaling pathway that involves phosphorylation of Cx43 at serine residue 373. Thus, LA causes an increase amount of Cx HCs at the cell surface and an increase in the Cx HC activity allowing more diffusion of Cx HC permeant ions and molecules across the cell membrane.

LA had previously been shown to increase the Cx HC activity after about 5 min [24], suggesting the involvement of signaling and/or metabolic steps that take time to occur. We found that LA requires the presence of a membrane transducer, GPR40, an Akt-dependent intracellular signaling pathway, and increased levels of Cx43 HCs in the plasma membrane, steps which could take some time to occur. The relevance of GPR40 is supported by three findings: 1) GW9508, an agonist of GPR40, also triggered an increase in Etd⁺ uptake that presented with a delay of ~5 min, suggesting the participation of the same pathway activated by LA, 2) the participation of other two receptors that can response to FFAs, GPR120 and CD36, was discarded, and 3) cells transfected with siRNA for GPR40 did not show LA-induced dye uptake. The latter result was specific because the Neg siRNA for GPR40 was ineffective at reducing the level of GPR40 or the LA-induced dye uptake. Interestingly, the effects of LA, a ω-6 long chain FFA, have been associated with GPR40 [25, 45]. In MKN28 cells, we found expression of GPR40 and GPR120, and CD36, but LA is known to be a

GPR40 endogenous ligand that induces activation through the Gq protein [19], which could explain the complete inhibition of LA on Cx HC activity in cells transfected with siRNA for GPR40, where we know that GPR120 expression was unchanged by siRNA-GPR40 (data not shown). Also, it is worth mentioning that the different receptors elicit differential response to FFAs, and in particular for GPR120 and CD36 it has been reported that they have no overlapping roles in intracellular signaling induced by FFAs [31].

The increase in Cx HC activity induced by LA was previously proposed to be PI3K- and Akt-dependent but independent of p38 kinase [24]. In the present work, we first confirmed that AKTi prevents completely the LA-induced dye uptake. Then, we found that serine 373 located in the carboxyl terminal of Cx43 is required for the LA-induced Cx HC response because HeLa-Cx43-S373A did not respond. Thus, Akt is likely to catalyze a later step in the GPR40 activation intracellular signaling pathway leading to changes in Cx43 HCs. Notably, it has been previously found that the size of gap junctions are greatly influenced by phosphorylation at Akt sites on Cx43 [36, 48, 49], suggesting that hemichannels and gap junctions share some aspects of their regulation. Interestingly, inhibition of Akt with AKTi in HeLa-Cx43 cells drastically reduced the Cx43 HC activity below basal level but did not reduce the levels of Cx43 in the cell membrane compared to control, suggesting that Akt-mediated phosphorylation might increase Cx43 plasma membrane levels and regulate other Cx43 HC features such as permeability and/or conductance. The fast opening of Cx HCs in cells exposed to DCFS is consistent with the involvement of a gating mechanism that increases the open probability of Cx HCs [40] without affecting Cx levels in the membrane [44]. In contrast, the LA-induced dye uptake required the participation of membrane and cytoplasmic events that increase the number of Cx HCs at the cell surface as shown by the increased levels of Cx43 detected in biotinylation of cell surface proteins followed by Western blot analysis.

The LA-induced increase in Cx HCs activity has been shown to be preceded by a small, transient increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) independent of Cx HCs, followed by a progressive increase in $[\text{Ca}^{2+}]_i$ that paralleled the Cx HC activity change [25]. In addition, the increase in $[\text{Ca}^{2+}]_i$ was prevented by chelation with intracellular BAPTA. However, it was not shown if the absence of divalent cations affects the LA-induced Cx HC activity. Here, we found that the LA-induced Cx HC activity is at least partially independent of the effect of reduced extracellular $[\text{Ca}^{2+}]$ since their effects at saturation were additive. Accordingly, regulation of Cx HCs by extracellular divalent cations has been shown to depend on amino acid residues located in Cx domains facing the extracellular milieu [4] while regulation by LA depends upon an intracellular transduction mechanism and modification of an amino acid residue located intracellularly in the C-terminal domain. The Etd^+ uptake induced by LA reached maximal values within 5 min making increased Cx43 protein synthesis an unlikely explanation for the change. Alternatively, the increase in Cx43 level in the cell membrane could result from increased trafficking of Cx43 from intracellular Cx43 stores, or less removal of Cx43 from the cell membrane or different distribution in raft membrane fraction. These mechanisms could lead to an increase in open probability due to the presence of more hemichannels. It remains to be demonstrated whether LA also affects the intrinsic open probability of hemichannels already present in the membrane.

Since LA increased the activity of hemichannels composed of Cx26 or Cx32 in HeLa cell transfectants and these Cxs were also found in MKN28 cells, it is likely that in the latter cells the Akt-dependent transduction mechanism that acts on Cx43 HCs also affects the activity of other Cx HCs. In support of this interpretation, knock down of GPR40 prevented the LA-induced increase in Cx26 and Cx32 HC activity. Moreover, the LA-induced Cx HC activity was completely abrogated in MKN28 cells pretreated with AKTi. Cx26 and Cx32 have been shown to be phosphoproteins [51-53], but it remains to be demonstrated whether phosphorylation of these Cxs is mediated directly by Akt or other protein kinases downstream of Akt. In cells that co-express Cx43 with other connexins (e.g., Cx26 and/or Cx32), it is possible that Cx43 HC opening promotes signal transduction pathways or alters the transmembrane ionic gradients triggering Cx26 HC and/or Cx32 HC opening.

In diverse cell types, increases in Cx HC activity have been associated with physiological and pathophysiological conditions [3], but epithelial cells of the gastrointestinal tract remained unexplored. Epithelial gastric cells likely see varying levels of FFA, including LA, thus it is likely that the activity of Cx HCs changes throughout the day. This regulation of Cx HCs might be important because they would favor the diffusional uptake of diverse small nutrient molecules that permeate hemichannels. Given the different properties of different connexins, the presence of different Cxs could allow the formation of homomeric (HC composed of one Cx type) and heteromeric hemichannels (Cx26 and Cx32 intermixed in a HC) with different permeability properties possibly favoring the diffusional exchange of different sets of small molecules [54]. However, the effects of LA have been associated mainly with inflammatory conditions [35, 55]. Since the cells expressing Cx43-S373A do not respond to metabolic inhibition, a proinflammatory condition that mimics hypoxia-reoxygenation [37], most likely the physiological or pathophysiological outcome of LA on cells via Cx HCs will depend on the intensity and duration of the stimulus; high concentrations or long term action of an effective LA concentration could lead to Ca²⁺ overload and thus degenerative mechanisms that activate an inflammatory response.

Our results lead us to propose that LA increases the Cx43 HC activity through activation of a signaling pathway that involves the membrane receptor GPR40 and AKT kinase activity. Then, the phosphorylation of Cx43 in serine 373 by AKT promotes an increase in the number of Cx43 HC at the cell surface, which increases the open probability of Cx43 HCs via a mechanism that is independent of extracellular [Ca²⁺].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Cx	connexin
DCFS	divalent cation-free solution
Etd⁺	ethidium
FFA	free fatty acid
GPCR	G-protein coupled receptor
GPR40	G-protein receptor 40
HC	hemichannel
LA	linoleic acid

Highlights

1. Linoleic acid increases the plasma membrane permeability of gastric epithelial cells
2. Activation of GPR40 receptor induces phosphorylation of connexin 43 at serine 373
3. Linoleic acid, via Akt, induces an increase in the amount of Cx43 at the cell surface

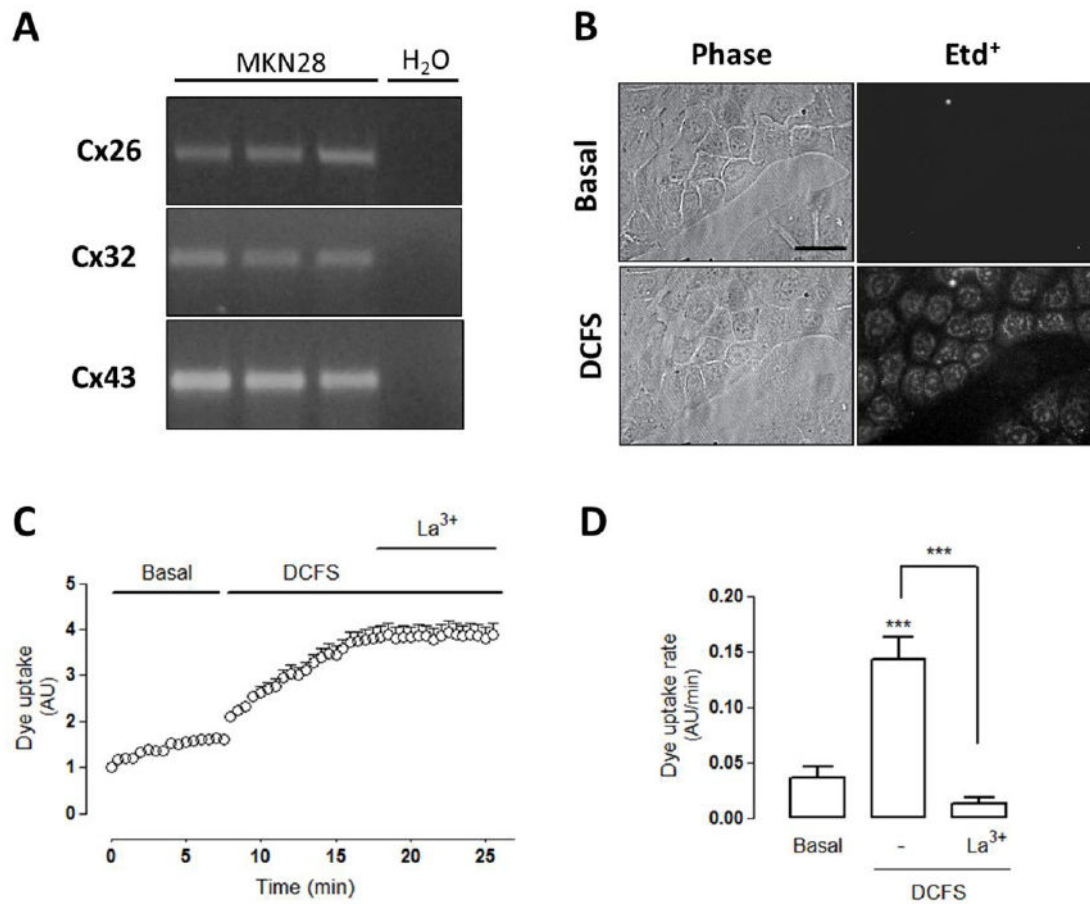


Figure 1. Gastric epithelial cells show connexin expression and hemichannel activity

(A) The mRNAs of connexins (Cxs) 26, 32 and 43 mRNA were detected by PCR (shown in triplicate). Negative H₂O controls are also shown. (B) Phase contrast images (left panels) show cells exposed to ethidium (5 μ M Etd⁺) for 5 min under (Top) basal conditions or (bottom) after exposure to divalent cation free solution (DCFS) for 5 min. Right panels show fluorescence of Etd⁺ (Scale bar: 50 μ m). (C) Representative Dye uptake curve under basal conditions and after exposure to DCFS followed by the application of lanthanum ions (La³⁺, 200 μ M). (D) Dye uptake rate of cells under the same conditions indicated in (C). Statistical significance versus basal conditions ***p<0.001 and versus DCFS ***p<0.001, n=3-6.

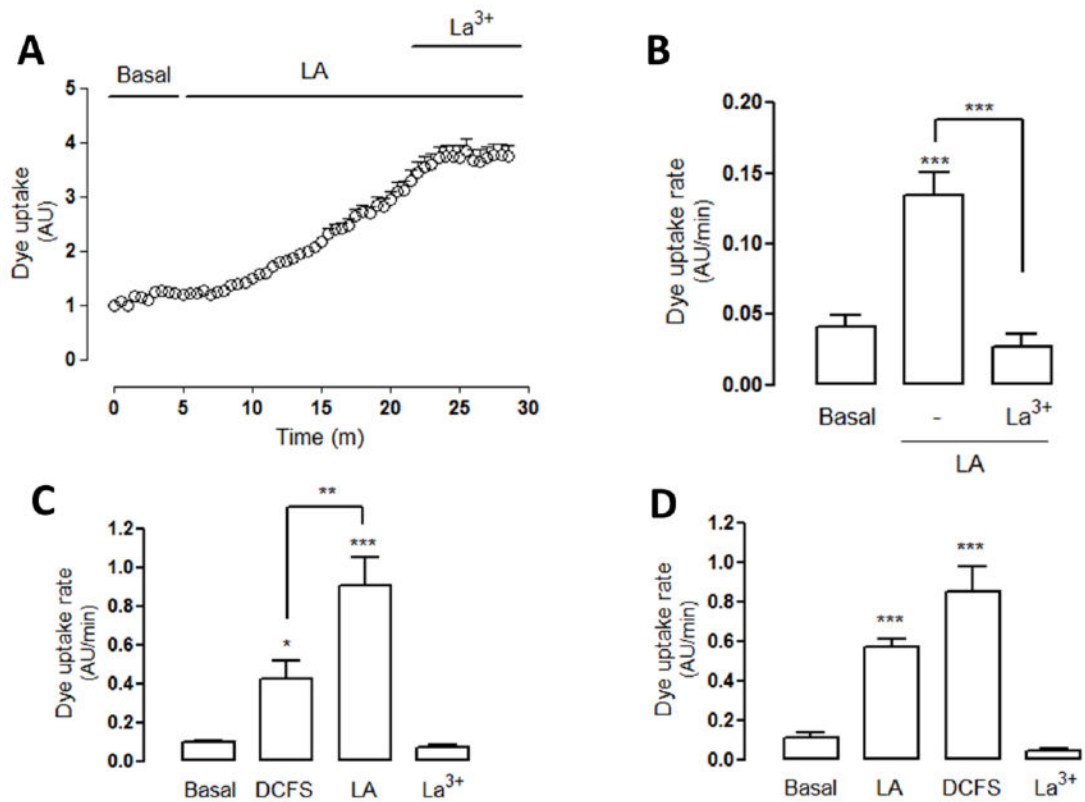


Figure 2. Linoleic acid increases hemichannel activity on MKN28 cells

(A) Representative dye (Etd⁺) uptake curve of MKN28 cells exposed to linoleic acid (LA, 100 μ M): ethidium uptake was increased ~5 min after treatment with LA. Dye uptake was blocked by lanthanum ions (La³⁺, 200 μ M). (B) Quantification of dye uptake rate of cells under basal conditions and treated with LA. The increase was sensitive to La³⁺. (C) Dye uptake rate of HeLa-Cx43 cells exposed first to divalent cation-free solution (DCFS) for 10 min followed by 100 μ M LA for additional 10 min and to lanthanum ions (La³⁺, 200 μ M) for 5 more minutes. (D) Dye uptake rate of HeLa-Cx43 cells exposed first to LA, then to DCFS, and finally to La³⁺ for the same periods of time as in (C). Statistical significance versus basal conditions *p<0.05, ***p<0.001, versus LA ***p<0.001 and versus DCFS **p<0.01, n=3-4.

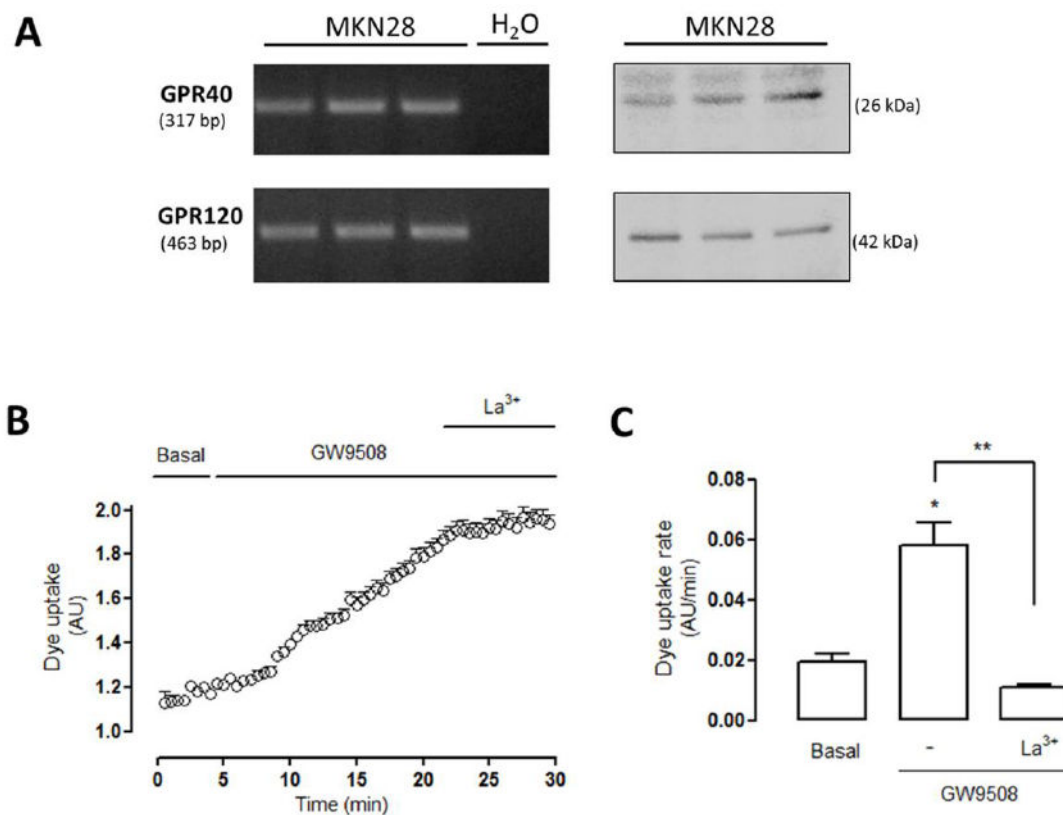


Figure 3. MKN28 cells express GPR40 and GPR120 and their activation increases hemichannel activity

(A) In total cellular homogenate of MKN28 cells the presence of GPR40 and GPR120 mRNA were detected by RT-PCR (H₂O was used as a negative control). Also, GPR40 and GPR120 proteins were detected by western blot analysis. The expected size of the amplicons and molecular weight of the proteins are indicated in base pairs (bp) and kilodaltons (kDa), respectively. (B) MKN28 cells were exposed to ethidium solution (5 μ M Etd⁺). Representative Dye (5 μ M Etd⁺) uptake curve of MKN28 cells exposed to 40 μ M GW9508, agonist by GPR40 and GPR120 receptors, followed by the application of lanthanum ions (200 μ M La³⁺). (C) Dye uptake rate under basal conditions and after consecutive application of GW9508 and La³⁺ as shown in (B). Statistical significance versus basal conditions *p<0.05 and versus GW9508 **p<0.01, n=3-4.

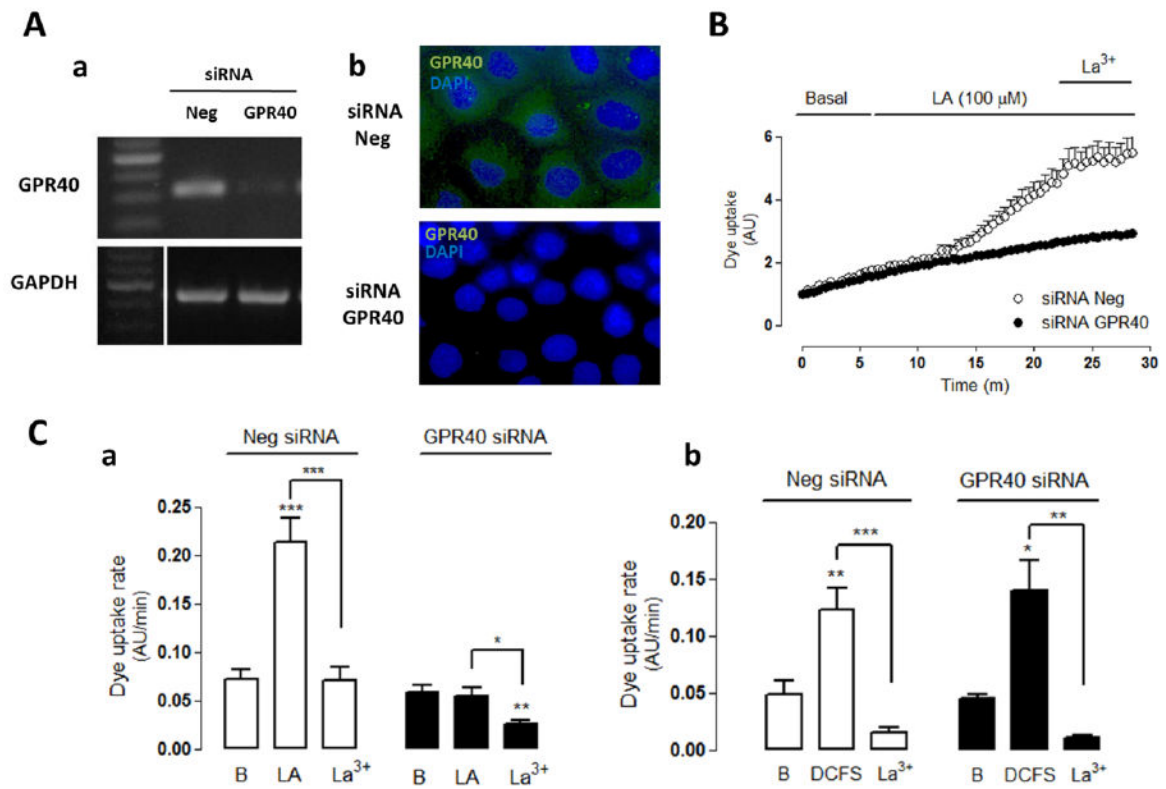


Figure 4. Knock-down of GPR40 receptor abrogates the hemichannel activity induced by linoleic acid

MKN28 cells were transfected with GPR40 siRNA or random sequence (Neg siRNA) and analyzed 40-48 h later. **(A)** The knock-down of GPR40 was confirmed by PCR (**Aa**) and immunofluorescence analyses (**Ab**). **(B)** Representative dye uptake ($5 \mu\text{M Etd}^+$) curve. In cells transfected with Neg siRNA the increased of dye uptake by linoleic acid (LA, $100 \mu\text{M}$) was abrogated by GPR40 knock-down (GPR40 siRNA). Under both transfection conditions, the LA effect was blocked by lanthanum ions (La^{3+} , $200 \mu\text{M}$). **(Ca)** Dye uptake rate in cells transfected with Neg or GPR40 siRNA under basal conditions and sequentially treated with LA and lanthanum ions (La^{3+}). **(Cb)** Dye uptake rate of cells transfected with Neg or GPR40 siRNA under basal conditions or exposed to divalent cation-free solution (DCFS) and La^{3+} as indicated. Statistical significance versus basal (B) conditions *** $p < 0.001$, versus LA * $p < 0.05$, *** $p < 0.001$ and versus DCFS $n = 4-5$.

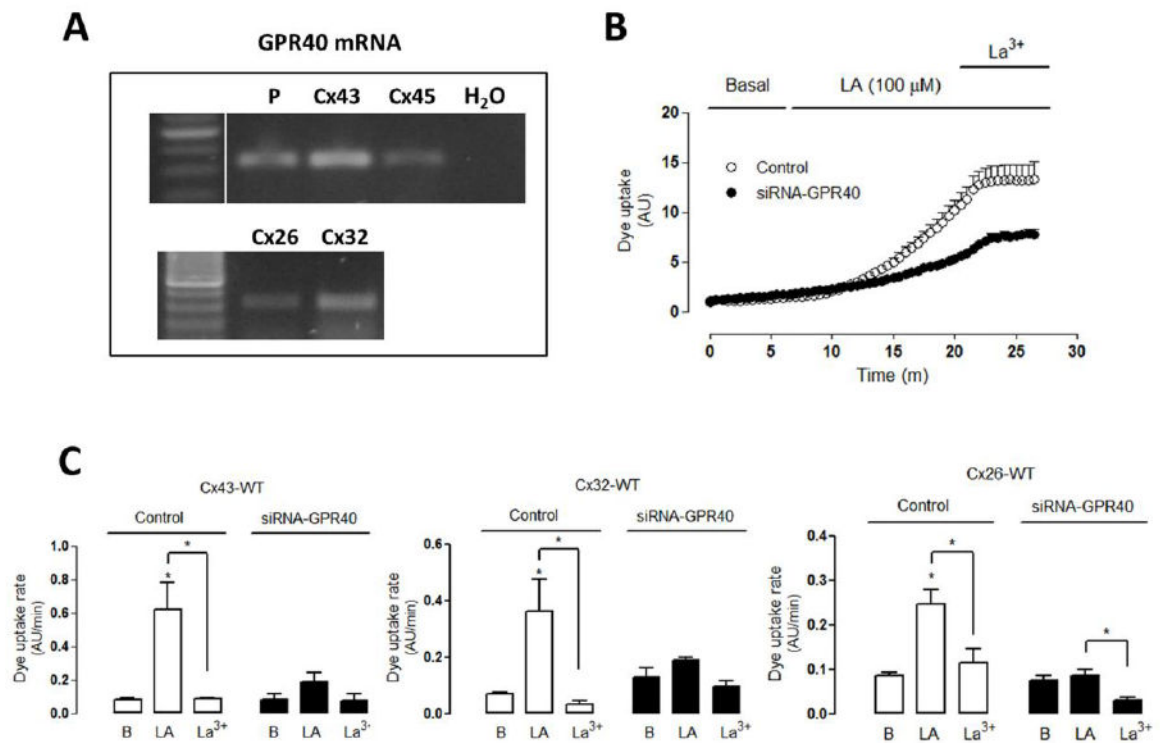


Figure 5. Linoleic acid induces GPR40-dependent hemichannel activity in connexin HeLa cells transfectants

HeLa cells stably transfected with connexins 43 (Cx43), Cx32 or Cx26 were used. Sister cultured cells were transiently transfected (40–48 h) with GPR40-siRNA. (A) The mRNA of GPR40 was evaluated in HeLa parental cells (P) or in HeLa cell transfectants (Cx43, Cx45, Cx26, Cx32). H₂O was used as negative control. (B) Representative Dye (5 μM Etd⁺) uptake curve of control HeLa-Cx43 cells and HeLa-Cx43 transfected with GPR40 siRNA treated with linoleic acid (LA, 100 μM) followed by treatment with 200 μM La³⁺. (C) Dye uptake rate of HeLa-Cx43, -Cx32 or -Cx26 cells under control conditions or transfected with GPR40-siRNA under basal conditions, followed by LA and La³⁺ treatment. Statistical significance versus basal (B) conditions *p<0.05 and versus LA*p<0.05, n=3-4.

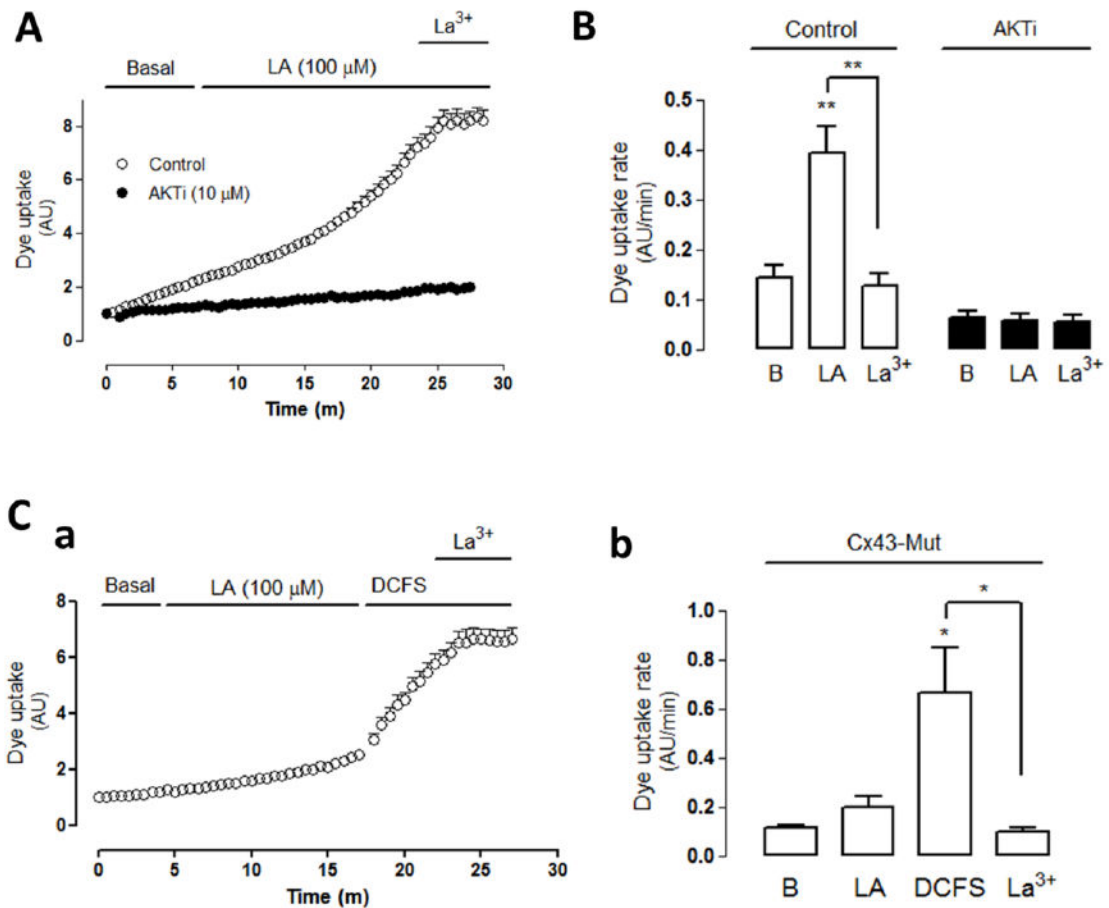


Figure 6. The linoleic acid-induced activation of connexin hemichannels is downstream of Akt (A) Representative dye (5 μ M Etd⁺) uptake curve of control cells treated with 100 μ M linoleic acid (LA) or cells pretreated with 10 μ M AKTi (Akt inhibitor) and then treated with LA. After 15 min with LA, cells were treated with 100 μ M lanthanum ions (La^{3+}) as indicated by the black traces on top of the graph. (B) Quantification of dye uptake rate under control conditions or after pretreatment with AKTi under basal conditions and sequentially treated with LA and LA plus La^{3+} . (C) Connexin43 with S373A mutation (Cx43-Mut) stably transfected into HeLa cells. (Ca) Dye uptake curve of cells exposed first to LA and then to divalent cation-free solution (DCFS) and DCFS plus 200 μ M La^{3+} . (Cb) Dye uptake rate of cells (Cx43-Mut) under each of the conditions described in (a). Statistical significance versus basal (B) conditions * p <0.05, ** p <0.01, versus LA ** p <0.01 and versus DCFS * p <0.05, n =3-4.

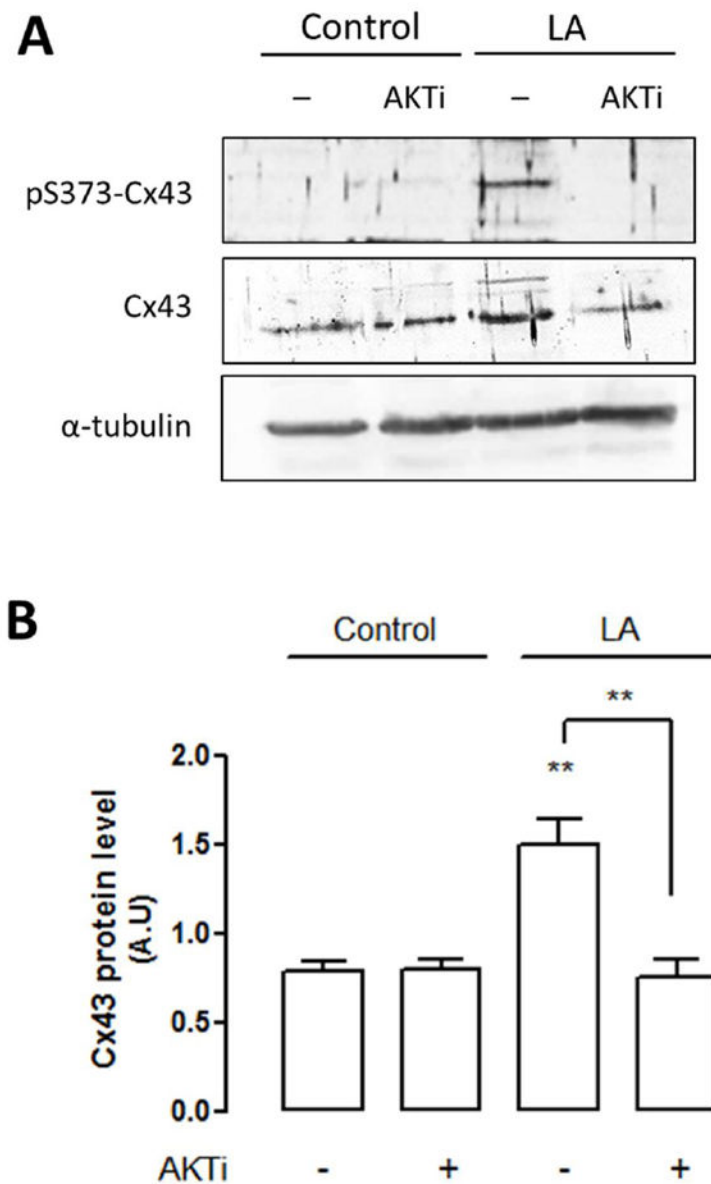


Figure 7. Linoleic acid increases the number of connexin43 hemichannels at the cell surface in HeLa cells

The relative amount of Cx43 at the cell surface in HeLa-Cx43 cells was determined by biotinylation of cell surface proteins followed by western blot assays. **(A)** Representative image of the relative amount of Cx43 at the cell surface in HeLa-Cx43 cells, in absence (control) or the presence of linoleic acid (LA, 100 μ M), and with and without AKTi (10 μ M). The biotinylation was followed by western blot analysis using antibodies against Cx43 (Cx43) (repeated 3 times) or Cx43 phosphorylated at S373 (pS373-Cx43) (repeated 2 times). α -tubulin corresponds to the total protein fraction, before purification of surface hemichannels and was used as a loading control. **(B)** Quantification of cell surface Cx43 levels in control HeLa-Cx43 cells, in absence (control) or the presence of LA, and with and without AKTi or cells exposed to AKTi (10 μ M) in the presence or absence (control) of

linoleic acid (LA, 100 μ M). Statistical significance compared to control in the absence of AKTi. ** $p < 0.01$ and versus LA (without AKTi) ** $p < 0.01$, $n = 2-3$.

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