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Inflammatory conditions induce gap junctional communication between rat Kupffer cells both *in vivo* and *in vitro*

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Abstract

Connexin43 (Cx43), a gap junction protein subunit, has been previously detected in Kupffer cells (KCs) during liver inflammation, however, KCs phagocytose cell debris that may include Cx43 protein, which could explain the detection of Cx43 in KCs. We determined that KCs express Cx43 and form gap junctions both *in vivo* and *in vitro*. In liver sections of animals treated with LPS, Cx43 was detected at ED2+ cells interfaces, indicating formation of GJ between KCs *in vivo. In vitro*, unstimulated KCs cultures did not form functional GJs, and expressed low levels of Cx43 that showed a diffuse intracellular distribution. In contrast, KCs treated with LPS plus IFN-γ, expressed a greater amount of Cx43 at both the, protein and mRNA levels, and showed Cx43 at cell-cell contacts associated with higher dye coupling. In conclusion, activation of KCs *in vivo* or *in vitro* resulted in enhanced Cx43 expression levels and formation of GJ that might play relevant roles during liver inflammation.

Keywords

connexins; liver; disease; macrophages; inflammation

Introduction

Kupffer cells (KCs), a class of resident mononuclear phagocytes, are situated in the liver sinusoids in close contact with endothelial cells, and play important roles in the hepatic immune response (1). Macrophages, including KCs, communicate with each other and with other cell types at least in two ways: 1) indirectly, using autocrine and paracrine signals, such as cytokines; and 2) directly, via cell adhesion molecules through ligand-receptor-like interactions (2). A less explored communication pathway is that which is mediated by gap junctions. A few reports have demonstrated the presence of gap junctions in immune cells, including monocytes/macrophages, microglia, polymorphonuclear cells, and dendritic cells (3). Nonetheless, the

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immune signals that regulates the expression of connexins (Cxs), protein subunits of gap junctions, and their role in KCs remains to be elucidated.

Gap junctions (GJ) are intercellular channels that allow communication between contacting cells by mediating reciprocal exchange of ions and small molecules, such as some second messengers (i.e., cAMP and IP_3) (3). Gap junction channels are formed by two hemichannels or connexons; each connexon is a hexamer of Cxs that are encoded by a gene family (3). The expression of Cxs and properties of these channels are affected by numerous extracellular factors and post-transcriptional modifications in a Cx- and cell type-dependent manner (3).

In a normal liver, most KCs are sparse but they migrate along sinusoidal walls (4) and small KCs aggregates (about 2–3 cells) are rather frequent (5). KCs develop multiple philopodia allowing them to establish more cell-cell contacts and form aggregates (5,6), in response to pro-inflammatory agents, such as bacterial endotoxin, lipopolysaccharide (LPS). Moreover, large KC aggregates located preferentially in perivenular regions of hepatic acini have been observed in alcoholic and non-alcoholic steatohepatitis (7). In favor of the hyphothesis that KCs might express GJ, previous studies have demonstrated enhanced Cx43 immunoreactivity in KCs of inflamed liver in rats treated with CCl_4 or LPS (5,8). Nevertheless, Cx43 immunoreactivity could have been acquired by phagocytosis of cell debris (i.e., dead endothelial cells) containing Cx43. Therefore, direct demonstration of Cx expression and GJ channels formation between KCs remains to be shown.

In this study, we demonstrate that KCs present in the liver of LPS treated rats form GJ plaques containing Cx43, and that primary cultures of KCs treated simultaneously with LPS and interferon-gamma (IFN-γ) express functional GJ that might coordinate KC responses during liver inflammation.

Experimental Procedures

LPS injection as a model of endotoxic shock

Sprague-Dawley rats (180–200 g body weight) obtained from the Animal Institute of the Pontificia Universidad Católica de Chile were used. LPS was administrated as previously described (5) with the approval of the Pontificia Universidad Católica de Chile bioethical committee. Briefly, rats after 6 h post intraperitoneal injection of LPS (*E.coli* serotype 0127:B8, 6 mg/kg body weight) were anesthetized (with I.P. injection of pentobarbital) and decapitated. As previously described, controls with vehicle injection did not show aggregation of KCs or enhanced Cx43 immunoreactivity (Fig. 1 A–D and (5)).

Isolation of KCs

Male Sprague-Dawley rats (180–200 g body weight) from the Animal Institute of the Pontificia Universidad Católica de Chile were used. Animals were sacrificed as described above. First, using a previously described technique, the liver was perfused with collagenase type I to obtain a cell suspension which was subjected to a double Percoll gradient to separate hepatocytes and KCs (5,9). Isolated KCs were then resuspended in Waymouth's medium; 2×10^6 cells per 3 ml of medium were preplated in 60 mm Primaria plates (Falcon, Becton Dickinson, NJ, USA). After 30 minutes, cells were washed 3 times with fresh medium to eliminate non-adherent cells. KC cultures were 99% pure as assayed by the number of ED-2+ cells.

Microinjection of LY (Dye coupling)

To evaluate the functionality of GJ channels between KCs, the tissue culture medium was replaced with bicarbonate-free F12 nutrient mixture containing 10 mM HEPES buffer, pH 7.2. Then, the intercellular transfer of LY (5% w/v in 150 mM LiCl) was evaluated by

microinjecting the dye into a single cell of a group of KCs. Dye transfer to neighboring cells was evaluated one minute later, as previously described (10). Cells were scored as coupled if dye transfer occurred to one or more adjacent cells. Dye transfer was evaluated using a Nikon Diaphot microscope equipped with a Xenon arc lamp illumination and a Nikon B filter block (excitation wavelength 450–490 nm; emission wavelength: > 520 nm). Four independent experiments were performed, in which a minimum of 20 cells were microinjected. Coupling was presented as incidence of coupling (%).

Immunofluorescence

Liver cryosections (80 μm thick, to reconstruct the aggregates of inflammatory cells, from normal and LPS injected rats) or cultures of KCs plated on coverslips, were fixed and permeabilized in 70% ethanol for 20 min at −20°C, rinsed three times with PBS, and then incubated in blocking solution (5 mM EDTA, 1% fish gelatin, 1% BSA and 1% horse serum) for 30 min at room temperature according to a previous published protocols (11–13). Samples were then incubated in primary antibody (anti-Cx43, anti-ED2 or rabbit preimmune serum, 1:2000, 1:500 or 1:200, respectively) overnight at 4°C. Then, they were washed four times with PBS and then incubated with FITC-conjugated goat anti-rabbit IgG (Fab fragments; 1:500, Sigma, St. Louis, MO) or Cy3-conjugated sheep anti-mouse IgG (1:300, Sigma) for 1 h at room temperature, followed by another rinse in PBS for 1 h. Samples were then mounted using antifade reagent with DAPI (Molecular Probes, Grand Island, NY) and examined by confocal microscopy using a Leica confocal (Leica AOBS laser Scanning Confocal Microscope). To analyze colocalization of ED-2 and Cx43, serial Z-sections were obtained and then integrated using a Leica software, NIH Image J and Voxx program.

Western blot analysis

Relative Cx43 levels were determined by immunobloting as described (14). Briefly, KC cultures were rapidly harvested using cold 10 mM Tris buffer, pH 7.4, containing protease and phosphatase inhibitors. Then, cells were lysed by sonication (Microson XL2005, Plainview, NJ) for 20 s and protein content of each cell lysate was determined. To reduce Cx proteolysis, samples containing 150–200 μg of protein were separated on the same day by SDS-PAGE and electrophoretically transferred to nitrocellulose sheets, followed by incubation in blocking solution. Then, blots were incubated with affinity purified rabbit polyclonal antibodies prepared 6 against-Cx43 was followed by incubation with anti-rabbit IgG conjugated to HRP, and antigen-antibody complexes were detected by ECL (Perkin Elmer, Boston, MA).

RT-PCR detection of Cx43 mRNA

RT-PCR for Cx43 was performed as described previously (14).

Statistical Analysis

Mean differences were tested by non-parametric Kruskal-Wallis analysis. If a significant H-value was obtained, and a value of $p<0.05$ was considered significant.

Results

Systemic LPS injection induces formation of gap junction plaques containing Cx43 in aggregates of KCs in the liver

Previously, we described increased Cx43 levels in total liver homogenates and Cx43 immunoreactivity in KCs of rat liver sections of animals subjected to inflammatory conditions (5,8). However, the microscopy resolution used in those studies did not allow detection of GJ plaques between KCs forming aggregates typical of liver inflammation (5,7,8). In the current report, we used three color confocal microscopy, 3D reconstruction and 80 μm thick tissue

sections to examine Cx43 immunoreactivity and colocalization with ED-2, a KC marker, in cells forming aggregates after systemic LPS injection.

In the liver of normal rats co-localization of Cx43 and ED2 was detected but was much less frequent than in LPS-treated animals (under control conditions: <20% ED2+ cells were Cx43 + and after 5–6 h of LPS administration ~80% ED2+ cells were Cx43+). In control animals, low Cx43 immunoreactivity (Fig. 1B) was detected around periportal venules (PP venule, see arrows), some in KCs (ED2 positive cells, Fig. 1 C and D). In contrast, in LPS-treated animals, small clusters of ~3–10 KCs (ED2+) were frequently found (reconstructed by confocal microscopy, Fig. 1 G and K) and intense Cx43 reactivity was mainly localized at cell-cell contacts (Fig. 1F and J, also see merge Fig. 1 H and L). Samples incubated with pre-immune sera did not show positive immunostaining (data not shown). Moreover, 5–6 h after LPS treatment the number of ED1+ cells (a pan-macrophage marker) negative to ED2 was less than 5% (n=5; not shown), suggesting that at that time there was little macrophages/monocytes infiltration. Thus, these results indicate that an *in vivo* inflammatory stimulus can induce the expression of gap junction plaques containing Cx43 in KC aggregates, commonly found in inflamed liver (5,7,8)

Treatment with LPS plus IFN-γ induces intercellular communication via gap junctions between rat KCs

Since previous studies in monocytes/macrophages and microglia have demonstrated induction of dye coupling after treatment with LPS plus IFN-γ (14,15), the possibility that these proinflammatory agents induce dye transfer between KCs was evaluated. The effect of these agents was tested on KCs isolated from normal rats plated and kept in culture for 2 hours before treatment with the proinflammatory agents. Cells were treated either with LPS (1 μg/ml) or IFN-γ (100 U/ml) or LPS plus IFN-γ (1 μg/ml and 100 U/ml, respectively). At time zero, LY microinjected into one cell did not spread to neighboring cells under each condition studied, revealing a very low incidence of dye coupling (≤5%) (Fig. 2A). The coupling index (number of coupled cells when dye coupling was positive) was 0.5±0.3 cells by microinjected cell. The addition of LPS or IFN-γ alone did not enhance the incidence of dye coupling during the time course studied (data not shown). But, the combined treatment with LPS plus IFN-γ resulted in a progressive increase in the incidence of dye coupling, reaching a maximal value of ~30% between 2 and 9 hours post-treatment (Fig. 2Aand B). In addition, the coupling index was 2.7 ±0.6 cells by microinjected cell. After either 2 or 9 h treatment, the subsequent addition of LPS plus IFN-γ did not further increase the incidence of dye coupling induced by the first treatment these pro-inflammatory agents (n = 9; data not shown).

In a separate set of experiments, the incidence of dye coupling induced by the co-application of LPS and IFN-γ was blocked by the acute treatment with 35 μM 18-α-glycyrrhetinic acid (AGA, n = 9, Fig. 2A), a GJ blocker (10,16). After blocker wash out (3 washes of 1 min each), rapid recovery to the original incidence of dye coupling was observed (Fig. 2A). Similar results of reversible GJ blockade were obtained with 500 μM octanol, another GJ blocker (n =5, Fig. 2B). DMSO (0.2%) and ethanol (0.2%), the AGA and octanol vehicles, respectively, did not reduce the incidence of dye coupling induced by LPS plus IFN-γ (n = 5; data not shown).

To rule out the possibility that microinjected LY might be released to the extracellular space and then incorporated to neighboring cells via organic anion transporters or pannexin1 hemichannels activated by a rise in intracellular free Ca^{2+} concentration through P2X channels (17), dye coupling assays were performed either in the presence of probenecid (18), an organic anion transporter blocker, or oxidized ATP, a P2X channel blocker (19). The dye transfer of LY to neighboring cells after microinjection of LY into a single cell was not altered by the presence of 50 μM probenecid or 100 μM oxidized ATP (for each compound, n = 4, Fig. 2C).

Therefore, GJ are likely to be the main, if not the only, pathway for intercellular LY transfer in KC cultures treated with LPS plus IFN-γ.

Proinflammatory agents induce redistribution of Cx43 to cell-cell contacts in KC cultures

To further analyze whether the induction of dye coupling by LPS plus IFN-γ treatment was associated with formation of gap junction plaques at KC interfaces, immunofluorescence confocal microscopy was performed. Under in vitro basal condition, KCs showed a diffuse cytoplasmic Cx43 staining (Fig. 3B). Treatment with LPS plus IFN-γ for 2–9 h induced a stronger fluorescent signal for Cx43 than untreated cells (compare Fig. 3D versus B) and localization of Cx43 at cell-cell interfaces was found as well (Fig. 3D and F). In addition, treatment with LPS plus IFN-γ induced cell shape changes from round to flat facilitating contacts between neighboring cells (Fig. 2A compare to C and E). Staining with preimmune serum or non-immune isotype antibody was negative (data not shown).

Treatment with LPS plus IFN-γ enhances Cx43 mRNA and protein levels

RT-PCR and Western blot analysis were performed to determine whether the increases in both dye coupling and Cx43 staining by immunofluorescence observed in KC cultures that were treated with LPS plus IFN-γ were associated with changes in Cx43 total mRNA and protein levels.

Amplification of Cx43 mRNA by RT-PCR (zero time, lane 0) was detected in untreated KCs. Treatment with LPS plus IFN-γ for 4 and 9 h resulted in an increased level of Cx43 mRNA while no changes in amplification were detected for β-actin (Fig. 3A). Sequencing analysis confirmed that both amplification products corresponded exactly to the expected Cx43 and β-actin sequences (data not shown). These results indicate that both the protein expression and mRNA production of Cx43 were up-regulated by LPS plus IFN-γ treatment. Treatment with LPS or IFN-γ alone did not affect the mRNA levels (n = 3; data not shown).

Western blots revealed low levels of non-phosphorylated Cx43 protein in untreated KC cultures (Fig. 3B). However, in cultures treated with LPS plus IFN-γ, levels of the non-phosphorylated (NP) and phosphorylated (P2 and P3) forms of Cx43 were higher than in control cultures. The density of all bands increased progressively after 1, 2, 4 and 9 h of treatment with both cytokines (Fig. 3B). Treatment with LPS or IFN-γ alone did not affect the levels of Cx43 (n= 3; data not shown).

DISCUSSION

This report describes inflammatory conditions that induce gap junctional communication between KCs. *In vivo*, treatment with LPS induced formation of Cx43 gap junction plaques between KCs, suggesting that they become functionally coupled. *In vitro*, gap junctional communication between KCs was induced by LPS plus IFN-γ treatment and was associated with increased Cx43 mRNA, protein, and presence of Cx43 protein in cell-cell contacts.

Our confocal data obtained from liver sections of rats injected with LPS indicate that before any significant infiltration of macrophages/monocytes occurs, Cx43 is present in KC clusters and is preferentially localized at cell-cell interfaces. This finding suggests that gap junctional communication between KCs can occur *in vivo*, and may be induced by the increased liver expression of proinflammatory factors produced during the endotoxic shock including TNF-α and IFN-γ (20). Our *in vivo* and *in vitro* results indicate that KCs express at least Cx43 but they might also express other Cx types as other macrophagic cells do. For example, monocyte/macrophages also express Cx37 (21) and Cx45 (19) and microglia also express Cx36 (22).

Further studies will be required to elucidate which other Cx types and under which conditions they are expressed by KCs.

While in KCs the upregulation of Cx43 is induced *in vivo* by LPS injection, in hepatocytes, inflammatory factors down regulate gap junction proteins. Cx32 and Cx26, two parenchymal cells Cxs, are reduced in acute liver inflammation induced by LPS (5,23–26), hepatic ischemia/reperfusion (25), and cholestasis caused by common bile duct ligation (5,27). Under these conditions the expression of Cx43 by KCs, non-parenchymal cells, is enhanced to clear/repair the damage. However, the dysregulation of parenchymal versus non-parenchymal Cxs, result in alterations in the liver structure, subsequently resulting in alterations in glucose secretion, bile secretion and glycogenolysis, known to be coordinated by gap junctions and intercellular propagation of calcium waves (10,28,29). The migratory phenotype and clustering of KCs have been associated with macrophage activation, phagocytosis, debris clearance and apoptosis of macrophages and damage parenchymal cells (30–32).

Our *in vitro* data, LPS plus IFN-γ, but not either proinflammatory agent alone, induced gap junctional communication in a similar extent as previously described for macrophages/monocytes and microglia (14,15). LPS plus IFN-γ treatment induced intercellular dye transfer in ~30% KCs and induced ~45% incidence of dye coupling in macrophages/monocytes and microglia (14,15). Similarly, peptidoglycan, another proinflammatory agent, induces dye coupling in ~45% microglia (33). In KCs, the percentage of intercellular coupling was not affected by a second addition of LPS plus IFN-γ, suggesting that maximal response was achieved. The induction of gap junctional communication in a fraction of macrophagic cells may be a consequence of the various differentiation stages of the cells. Kupffer cells show functional heterogeneity which, like hepatocytes, is contingent upon their position in the liver acinus (30). For example, KCs in the periportal area are highly sensitive to cytokines that trigger an inflammatory/phagocytic phenotype, while the KCs in the centrilobular area are more cytotoxic/regulatory (34). Differences in cell surface receptor densities might explain why only a fraction of cells respond to LPS plus IFN-γ. In agreement with this interpretation, treatment of microglia with a Ca^{2+} ionophore that bypasses membrane receptors induces a much higher incidence of gap junctional communication (~75%) (35) as compared to LPS plus IFN-γ (~45%) (15).

The presence of GJ channels in immune cells has been associated with activation and coordination of specific events, such as transmigration, protease secretion, antigen presentation and cell differentiation (14,15,33,35–38). All these functions require cell specific activation that is driven by the indirect action of soluble inflammatory factors. Although all cells would be exposed to the same stimulus present in the extracellular milieu not all of them necessarily express the same surface receptor density and therefore the response is not the same in all responsive cells. This putative limitation could be overcome by direct cytoplasmic communication between contacting cells providing a direct and specific transfer of activation signals only between cells coupled to the primary activated cells.

Our current data support our overall hypothesis that gap junctions between immune cells allow an efficient immune response by coordinate intercellular signaling (3), especially under pathological conditions, such as septic shock, cholestasis, cirrhosis, AIDS/HIV-1, ischemia/reperfusion and liver cancer, where elevated inflammatory cytokine levels have been detected. In conclusion, here we demonstrated that KCs express functional gap junctions *in vivo* and *in vitro,* suggesting that these intercellular channels might be a good target to reduce the inflammatory response during hepatic disease.

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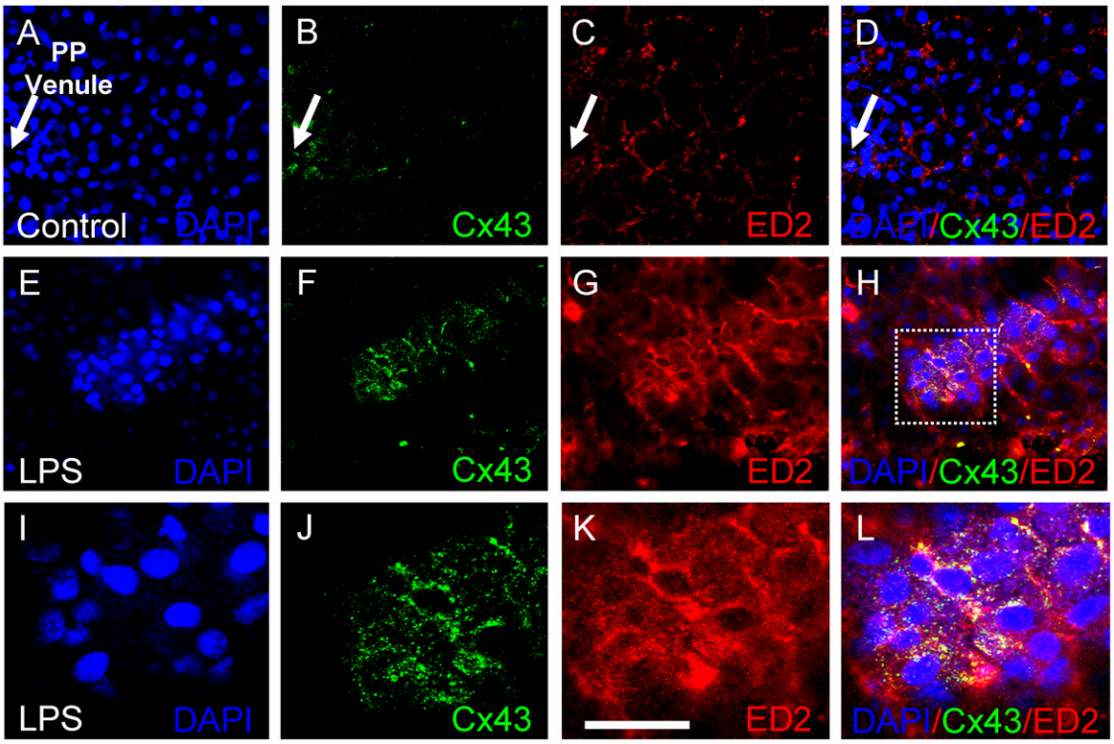

Figure 1. LPS administration induces formation of gap junction plaques between KCs in the liver

Immunohistochemistry and confocal microscopy of liver sections from normal and LPS injected rats was performed to analyze distribution and levels of Cx43 immunoreactivity in ED2 positive cells (KCs). Panels A, E and I, correspond to DAPI nuclear stain. (A–D) In normal liver sections few KCs (ED2+ cells) were positive to Cx43. (E–H) Liver sections obtained from rats injected with LPS 24 h before sacrifice show aggregation of KCs with strong signal for Cx43 at cell-cell appositions. I to L, are enlargements of the same area shown in E–H located within the dotted square illustrated in H, to show colocalization (yellow signal) of both Cx43 and ED2. PP venule: periportal venule are indicated by arrows. Bar: 150 μm for A–H and 35 μm for I–L.

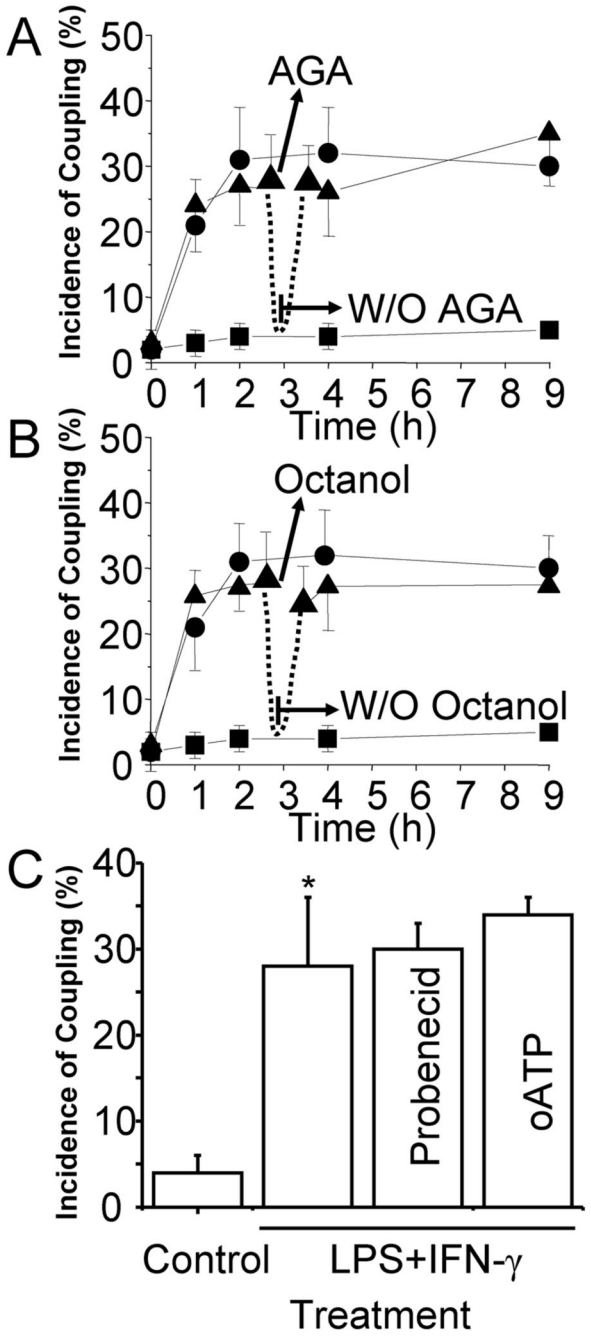

Figure 2. Treatment with LPS plus IFN-γ induces gap junctional communication and redistribution of Cx43 to cell-cell contacts between rat KCs

(A) The incidence of dye coupling (LY) was evaluated in cultures of rat KCs under control conditions and after LPS plus IFN-γ treatment. The incidence of coupling in control conditions was ~5 % (▪). In one set of experiments, treatment with 1 μg/ml LPS plus 100 U/ml IFN-γ induced a progressive increase in incidence of dye coupling (●). In a second set of experiments, the dye coupling induced by LPS plus IFN-γ was sensitive to the acute application of 18-α-glycyrrhetinic acid (AGA, 35 μM) or octanol (500 μm, Graph **B**) in a reversible way after washout (W/O) of the blocker (▲, see doted line). Each point corresponds to the average of 9 (AGA, Graph A) or 5 (Octanol, Graph B) experiments, in which a minimum of 20 cells were

scored. **(C)** Dye coupling induced by LPS plus IFN-γ between KCs, was not altered by treatment with probenecid (50 μM), an organic anion transporter blocker, or oxidized ATP (oATP, 100 μM), a P2X channel blocker, suggesting that the dye coupling observed was due to gap junctions channels and no other channels permeable to LY, such as organic anion transporters or P2X ATP receptors (n=4, * $p<0.005$). Each time point corresponds to the average ± SD.

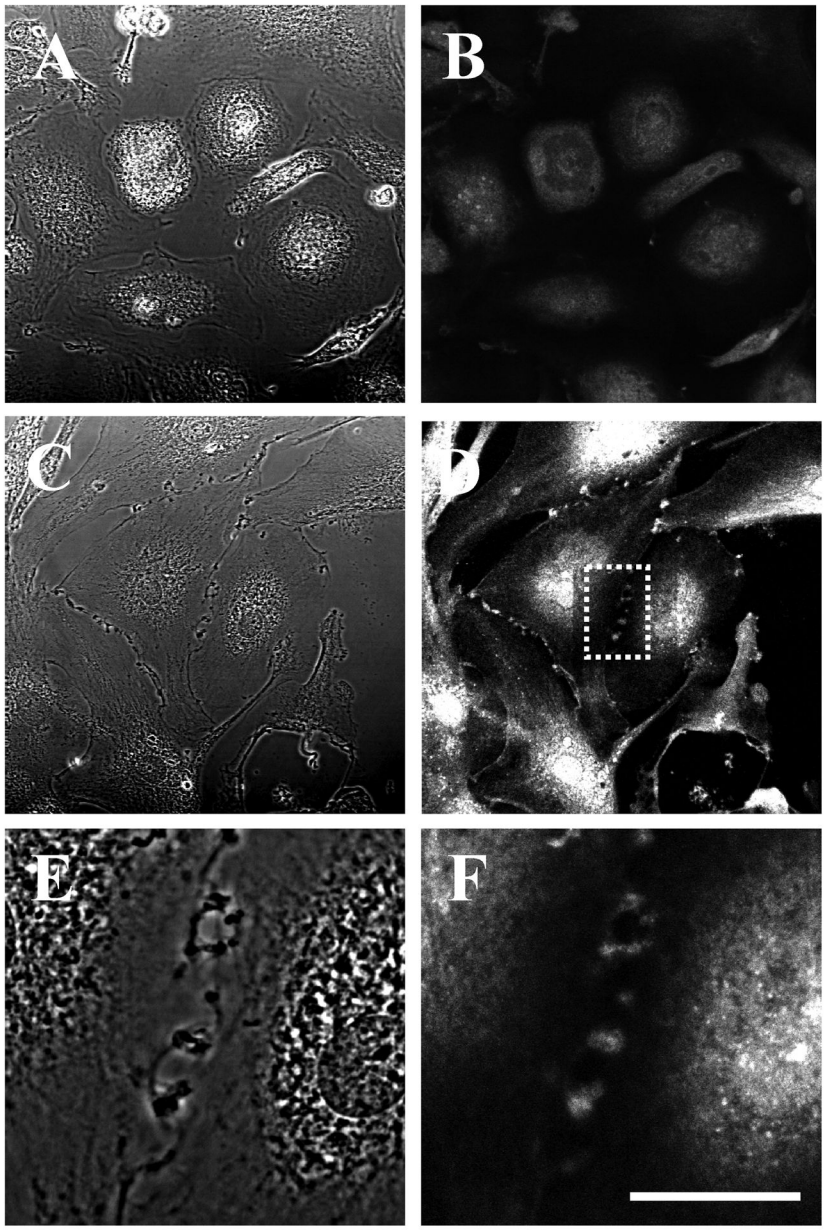

Figure 3.

Immunofluorescence for Cx43 (with anti-Cx43 F_{ab} antibody) of rat KCs in culture in control and after 2 h of treatment with LPS plus IFN-γ. A, C and E, are phase contrast views of the fluorescent fields shown in B, D and F, respectively. Under control conditions the distribution Cx43 was mostly perinuclear (B). However, after LPS plus IFN-γ treatment strong Cx43 reactivity was detected at cell-cell appositions (D and F). E and F, are enlargements of the area indicated in panel D (dotted square). No immunostaining with preimmune serum was detected (data not shown), Bar: 70 μm for A–D and 8 μm for E and F.

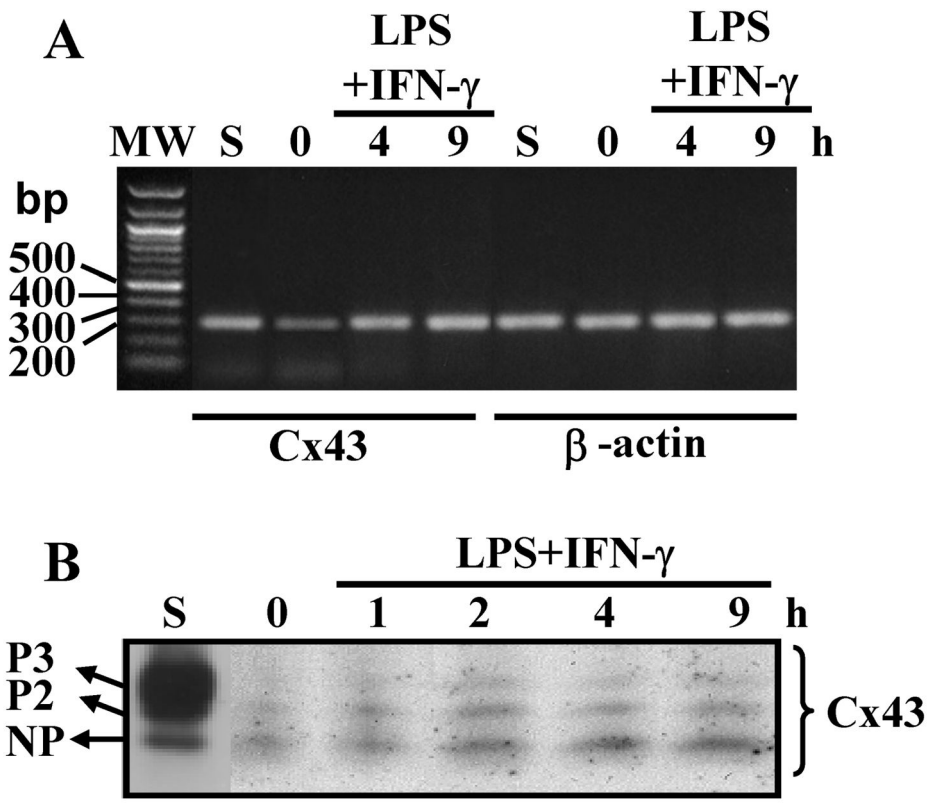

Figure 4. Treatment with LPS plus IFN-γ increases the levels of Cx43 protein and mRNA in KC cultures

(A) The relative levels of Cx43 mRNA were measured by semiquantitative RT-PCRs. Ethidium bromide staining shown as a band of 291 bp (arrow in the upper gel) corresponds to the amplification product obtained using Cx43 specific primers and total RNA extracted from KC cultures treated with LPS plus IFN-γ for 4 and 9 h. β-actin was used a loading control and their amplification resulted in a amplification product of 281 pb. MW corresponds to 100 bp DNA ladder (n=3). (B) Cx43 protein levels were determined by Western blot analysis from KC lysates (150 μg of protein per lane) using a rabbit anti-Cx43 antibody. Connexin43 levels were analyzed at zero time or after 1 μg/ml LPS plus 100 U/ml IFN-γ treatment for 1, 2, 4 and 9 h. An aliquot of rat heart (30 μg of proteins) was used as positive control for Cx43 (lane S). The unphosphorylated (NP) and two phosphorylated forms of Cx43 (P2 and P3) are indicated on the left side of the immunoblot.