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Induction of lactoferrin gene expression by innate immune stimuli in mouse mammary epithelial HC-11 cells

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Abstract

Lactoferrin (LF) is a multifunctional protein. While its functions and mechanism of actions are actively being investigated, the cellular signals that regulate LF expression have not been as explored. We have previously demonstrated that LF is upregulated by estrogen in reproductive system. In this study, we show that the expression of LF was stimulated by bacterial lipopolysaccharide (LPS) and double-stranded RNA (dsRNA) in normal mouse mammalian HC-11 cells. When cells were exposed to either LPS or dsRNA, the mRNA and protein of LF were increased in a dose- and time-dependent manner, yet the kinetics of LF induction by dsRNA or LPS was different. The LPS and dsRNA-induced LF was mainly released into the culture medium where it blocked TNF- α production in exposed cells. We explored the mechanisms of LF induction by LPS and dsRNA using specific inhibitors and found that the induction could be attenuated by inhibitors to PKC, NF- κ B, p38 and JNK, but not by an inhibitor to PKA. Interestingly, ERK inhibitor was effective against dsRNA but not against LPS induction of LF. These data suggest that LF was induced by LPS and dsRNA through PKC, NF- κ B and MAPK pathways which in turn plays an inhibitory role in the continuation of innate inflammation.

Keywords

lactoferrin; LPS; dsRNA; HC-11 cells; PKC; MAPK; NF- κ B; inhibitors

1. Introduction

Lactoferrin, a member of the transferrin family, was originally found in milk as an iron binding glycoprotein. It is abundant in the secondary granules of the neutrophils and in biological fluids such as tears, saliva, seminal plasma and secretions of nasal, pancreatic, gastrointestinal, bronchial and uterine tissue (see review and reference therein [1]). Lactoferrin serves as a barrier with well established roles in anti- bacterial, anti-viral (review and reference therein [2]) and anti-fungal [3] infections. It is also known to modulate immune responses during infections (review and reference therein [4]). The level of lactoferrin in milk, external secretion and serum significantly increases during infections suggesting that this protein plays an important role in host-defense against infectious agents. At the infection site, rapid response of the innate immune system leads to the influx of neutrophils that release lactoferrin from

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their secondary granules which contribute to the levels both locally and in the circulation [5]. Additionally, during infection with T-cell leukemia virus type 1 (HTLV-1) and bacteria, mammary epithelial cells have been reported to be a source of secreted lactoferrin [6,7]. Therefore, increased recruitment of neutrophils and compliment with an increase of lactoferrin production by epithelial cells occurs in the initial phase of infection. Although there has been extensive research efforts on the structure-function relationship of LF and its application in nutritional supplement and prevention of infections and cancers [8-12], studies on the regulation of LF expression by physiological stimuli has been limited [13,14].

The LF promoter was characterized and found to possess both constitutive and inducible elements [1,15]. Therefore, LF is not only constitutively expressed in the mucosal membranes and developmentally regulated in neutrophil granules, it is also upregulated by estrogen through a well characterized COUP/ERE element located -365 bp from the initiation start site [16,17]. In addition to the COUP/ERE, a GC-rich sequence located at -75/-40 in the mouse LF gene promoter was characterized as the mitogen response module (MRU), which responded to forskoline and epidermal growth factor (EGF) stimulation via protein kinase C (PKC) and protein kinase A (PKA) pathways [18,19]. Furthermore, an inflammatory response element was identified in the promoter of bovine LF gene [7]. Nonetheless, the mechanism of induction of LF gene expression by innate immune stimuli in mammary gland cells has not been reported.

LPS, a major component of the outer membrane of gram-negative bacteria, and dsRNA, a viral mimetic, induce a potent inflammatory reaction by activation of several intracellular signaling transduction pathways and leads to the expression of proinflammatory cytokines and chemokines [20-23]. Addition of LPS or dsRNA to the cells rapidly induces phosphorylation of mitogen-activated protein kinase (MAPK) pathways, p38 and c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK). In addition, LPS and dsRNA signaling leads to downstream NF- κ B activation in several cell lines [24-27].

In this study, we examined the effects of LPS and dsRNA on the LF expression in normal mouse mammary epithelial HC-11 cells. We demonstrated that the LF was produced and secreted into medium in response to LPS or dsRNA stimulation where it blocked the activity of LPS and dsRNA in inducing TNF- α . We also explored the signalling pathways that are involved in the LPS and dsRNA induction of LF expression.

2. Materials and Methods

2.1. Reagents

Lipopolysaccharides (LPS), dsRNA poly (I:C), 17 β -Estradiol (E2), diethylstilbestrol (DES), calphostin C (PKC inhibitor), 8-Bromoadenosine-cAMP, Rpisorner (PKA inhibitor) and SP 600125 (JNK inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). IKK inhibitor VII (NF- κ B inhibitor), PD 98059 (ERK inhibitor) and SB 203580 (p38 inhibitor) was purchased from Calbiochem (San Diego, CA).

2.2. Cell line and tissue culture

Mouse mammary epithelial HC 11 cell line (kindly provided by Dr. D. Kerr) was grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 50 μ g/ml gentamycin, 10 ng/ml EGF and 5 μ g/ml insulin. Before the experiments, cells were seeded in 6-well plate (8×10^5 cells/well) overnight, the medium was then changed to serum-free medium and the cells were treated with LPS or dsRNA for various concentration or different times as indicated in the Figures and Legends.

2.3. RNA extraction, conventional PCR (RT-PCR) and quantitative real time PCR (q-PCR)

Total RNA was extracted by using either Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) or TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed using superscript reverse transcriptase (Invitrogen, Carlsbad, CA). For the RT-PCR, the amplifications were performed with the following protocol: 94°C for 2 min. followed by 28 cycles of 94°C for 30s and 72°C for 30s. The products were detected by using a 1.5% agarose gel. The q-PCR measurements were conducted by Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Amplifications were performed with the following protocol: 95°C for 3 minutes followed by 30 cycles of 94°C for 10s and 60°C for 30s. GAPDH was used as a housekeeping gene. The sequences of primers used in RT-PCR and q-PCR were as follows:

for mouse LF, forward primer 5'-AAACAAGCATCGGGATTCCAG-3' and reverse primer 5'-ACAATGCAGTCTTCCGTGGTG-3'; for mouse TNF- α , forward primer 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse primer 5'-TGGGAGTAGACAAGGTACAACCC-3'; for mouse IL-1 β , forward primer 5'-CAACCAACAAGTGATATTCTCCATG-3' and reverse primer 5'-GATCCACACTCTCCAGCTGCA-3'; for mouse IL-6, forward primer 5'-TTGCCTTCTTGGGACTGATGCT-3' and reverse primer 5'-GTATCTCTCTGAAGGACTCTGG-3'; for mouse GAPDH, forward primer 5'-TGCACCACCAACTGCTTAG-3' and reverse primer 5'-GATGCAGGGATGATGTTTC-3'. Each sample was quantified against its GAPDH transcript contents, and then normalized with the control group. The experiments were repeated three times and results are presented as fold of increase $\pm$ S.E.

2.4. Antibodies and western blot analysis

Anti-mouse LF serum (8345) was prepared as described previously [28]. Anti-GAPDH polyclonal antibody (FL-335) was purchased from Santa Cruz (San Diego, CA). Whole cells lysates were prepared in 1x Laemmli sample buffer (Bio-rad, Hercules CA) containing 2.5% 2-mercaptoethanol (ME). Nuclear and cytosolic extracts were prepared by using BD TransFactor Extraction Kits according to the supplier's protocol (BD Biosciences, Palo Alto, CA). After heating the samples at 95°C for 5 min, they were loaded on a 10% SDS-PAGE gel and separated by electrophoresis. The protein was then electrotransferred onto nitrocellulose membranes for immunodetection with lactoferrin antibody and the immunoreactive product were detected by the ECL system (Amersham Pharmacia, Piscataway, NJ). Expression of GAPDH was used as a control.

2.5. Confocal microscopy

Cells were grown on Lab-Tek chamber slides (NUNC, Rochester, NY) overnight and then fixed with 4% Para-formaldehyde for 30 min, permeabilized with 0.5% Triton X-100 in PBS for 1 h. The cells were treated with the serum blocking solution (Zymed laboratories Inc., South San Francisco, CA) for 2 h to block the nonspecific sites before immunostaining. For staining, the LF antibody was applied overnight and then incubated with Alexa Fluor 488 anti-FITC rabbit IgG antibody (Molecular probes Inc., Eugene, OR) for 2 h. The location of LF was visualized with a confocal microscopy (Zeiss LSM-510 equipped with an argon-krypton laser) using a 40 $\times$ 1.2 objective lens. Images were acquired on the microscopy at 512 $\times$ 512 resolutions over a 32s exposure period.

2.6. Mouse LF protein, conditioned medium and inhibitor studies

Mouse LF protein was prepared as described [28]. For preparing the conditioned medium, cells were plated and cultured in 10% FBS RPMI 1640 medium. The next day, the medium was

changed to FBS-free RPMI 1640 medium and the cells were exposed to 100 ng/ml LPS for 2 h. The medium was then removed and the cells washed with PBS once. Fresh FBS-free RPMI 1640 medium was added and continued to culture for 24 h. The medium was collected from these cells and designated as conditioned medium (CM) which containing the LPS-induced LF for further experiments. For the inhibitor study, cells were pre-treated with the inhibitors for 1 h and then treated with the different concentrations of LPS or dsRNA for the different time points.

3. Results

3.1. LPS and dsRNA induces LF expressions in the HC-11 mammary epithelial cells

The HC-11 mouse mammary gland cell line, originally cloned from the COMMA-D1 cell line from a mid-pregnant BALB/c mouse [29], retains many of the normal features of mammary gland epithelial cells [30] including intact Toll-like receptor-mediated signaling pathway [7]. Lactoferrin is expressed in the HC-11 cells and responded to estrogen stimulation (Fig. 1A) which is in agreement with our previous finding that lactoferrin is an estrogen target gene (review and reference therein, [1]). Using confocal microscopic examination, we found that immuno-reactive lactoferrin is mainly localized in the cytoplasm with few in the perinuclear region of the HC11 cells (Fig. 1B). To examine the effect of LPS and dsRNA on the lactoferrin expression, we treated the cells with various concentrations or with different length of time of LPS and dsRNA (Fig. 2). Levels of lactoferrin mRNA was quantified by q-PCR and the protein detected by western blotting analysis. We found that LPS strongly induced the expression of LF mRNA and protein in a dose-dependent (Fig. 2A) and a time-dependent (Fig. 2B) manner. The LF induction could reach more than 100 fold above control. Like the LPS, dsRNA also induces LF expression in a dose- and time-dependent manner (Fig. 2C and 2D, respectively). At the concentration of 1 µg/ml, dsRNA induced LF mRNA 5-fold above the control in 24 h and reached to a 14-fold peak at 10 µg/ml concentration (Fig. 2C). In addition, LF mRNA was rapidly induced within 1.5 h, 10 µg/ml concentration and reached the peak of expression in 6 h followed by a slow decline (Fig. 2D). As noted, the kinetics of LF induction by dsRNA and LPS was different with rapid induction by dsRNA and slow induction by LPS exposure (compare Fig. 2B and 2D). These data demonstrated that the HC-11 cell line could serve as a model to evaluate the regulation of lactoferrin expression by innate immune stimuli.

3.2 Induction of proinflammatory cytokines by LPS and dsRNA in HC11 cells

In response to LPS, epithelial cells produces proinflammatory cytokines which attract and recruit the immune response cells such as neutrophil, macrophage, T lymphocyte, etc to the infection site and to destroy or contain the invaders. To investigate the relationships of LF and proinflammatory cytokine expression after LPS exposure, we examined the expression pattern of LF and three important proinflammatory cytokines, TNF- α , IL-1 β and IL-6 in a time-dependent manner. Cells were treated with 100 ng/ml LPS for different length of times and the levels of LF, TNF- α , IL-1 β and IL-6 mRNA were measured by RT-PCR (Fig. 3A) and q-PCR (Fig. 3B and 3C). TNF- α , a key proinflammatory cytokine was dramatically induced within hours after LPS exposure (Fig. 3A and 3B). The induction was strong but transient for it reduced to low levels by 6 h after LPS exposure. IL-1 β was induced in 3 h by LPS but was less robust than TNF- α . Additionally, the induction remained at a lower level longer than TNF- α (Fig. 3A and 3C). IL-6, which is constitutively expressed in HC-11 cells, was increased approximately 3-fold by LPS and remained at this level for 24 h (Fig. 3A). The effect of dsRNA on TNF- α expression in the HC-11 cells was also examined. Like LPS, dsRNA induced a robust but transient expression of TNF- α within an hour after exposure (Fig. 3D). Induction of these proinflammatory cytokines by both LPS and dsRNA was an early event LF induction required a longer length of exposure (more than 3 h for LPS and 1.5 h for dsRNA). Although a longer

exposure time was needed for initial induction, LF expression remained high for 24 h after exposure (Fig. 2).

3.3 LF inhibits the induction of TNF- α by LPS and dsRNA

The significance of LF expression in response to innate stimuli exposure was investigated. After exposure, we found that a major portion of induced LF was released into the medium as demonstrated by Western blotting of the LF in the medium after LPS (Fig. 4A, left panel) and dsRNA exposure (Fig. 4A, right panel). The remaining LF in the cell was mainly associated with a crude nuclear extract fraction with very low levels as detected in the cytosolic fraction shown by Western blotting (Fig. 4B). This observation was supported by confocal microscopy (Fig. 4C) as most of the cells exposed to LPS are immunostained by the mouse LF antiserum negatively at the cytosol and positively around the perinuclear region.

It is known that LF blocks LPS' activity through multiple mechanisms [2]. We examined whether LPS-induced LF from HC-11 cells would also inhibit LPS' ability to induce TNF- α in this cell type. We first exposed the HC-11 cells to LPS (100 ng/ml) for 2h, the medium was removed and the cells were washed once with PBS. A fresh FBS-free medium was added to the pre-exposed cells for additional 24 h, the medium was collected and designated as "conditioned medium". It is expected that the conditioned medium would contain LPS-induced LF but would be devoid of LPS and of the majority of the rapidly induced proteins including TNF- α . When naive HC-11 cells were cultured with the conditioned medium, LPS was unable to induce TNF- α expression while those cells cultured in normal medium were not inhibited (Fig. 5A). This observation was verified by the addition of various concentrations of exogenous LF (10-200 ng/ml) and by a constant amount of LPS (10 ng/ml) to HC-11 cells (Fig. 5B). The ability of LPS to induce TNF- α was completely blocked by the presence of 50 ng/ml of purified mouse LF [28]. A similar experiment was conducted with dsRNA exposure. Under the experimental condition described earlier, various concentrations of purified mouse LF protein (1-10 μ g/ml) were added together with the dsRNA (1 μ g/ml) to the HC-11 cells for 2 h. Total RNA of the treated cells was prepared and the TNF- α mRNA levels were determined by q-PCR. The results showed that presence of 1 μ g/ml of LF reduced TNF- α mRNA by 70%. At 10 μ g/ml of LF, dsRNA-induced TNF- α was blocked by 90% while the same amount of LF alone had no effect on TNF- α expression (Fig. 5C, last column). These results suggest that LF induced by either LPS or dsRNA can inhibit the innate immune stimuli's activity.

3.4 Effect of inhibitors to PKC, PKA, NF- κ B and MAPK on the LPS and dsRNA induction of LF

LPS binds to the Toll-like receptor 4 (TLR4) and activates multiple signaling pathways which leads to the induction of multiple immune response genes [31-33]. To investigate which pathway(s) leads to the induction of LF and TNF- α by LPS, we used various inhibitors that blocked kinase activities required for different signaling pathways. Cells were pre-treated with inhibitor for an hour followed by the LPS exposure for 3h. We found that the PKC inhibitor, Calphostin C, completely blocked the LF expression (Fig. 6A, left panel) but had no inhibitory effect on TNF- α (Fig. 6A, right panel). Interestingly, an inhibitor of PKA (Rp-8-Br-cAMP) had no effect on the LPS induction of either the LF or TNF- α gene. The NF- κ B pathway is a well established pathway for LPS [24,25]. As expected, blocking the pathway with an inhibitor to IKK (VII) completely blocked both LF and TNF- α expression. The real time PCR data was supported by Western blotting where LF protein production was also severely reduced (Fig. 6A, lower panel). Signaling pathways of LF induction by dsRNA was also investigated with the specific inhibitors. Similar to LPS, induction of LF by dsRNA was blocked by inhibitors of PKC and NF- κ B but not by PKA (Fig. 6B). The inhibitory effect was also seen by the reduction of dsRNA-induced LF protein through Western blotting (Fig. 6B, lower panel).

The three major MAPK pathways were also investigated by studying inhibitors to ERK (PD 98059), p38 (SB 203580) and JNK (SP 600125) activity. Cells were pre-treated with these inhibitors for 1 h, followed by the addition of LPS for another three hours. The expression of LF and TNF- α were then determined by q-PCR (Fig. 7A). The results demonstrated that the p38 and JNK inhibitors, but not the ERK inhibitor reduced LPS- induced LF mRNA levels by 70% (from 18 to 6 fold). Interestingly, the three MAPK inhibitors did not inhibit but enhanced the LPS-induced TNF- α expression under the same experimental conditions (data not shown). The inhibitors that blocked all three major MAPK pathways also inhibited the induction of LF by dsRNA. This is in contrast to the LPS induction pathways which were blocked only by two inhibitors, the SB 203580 for p38 and the SP 600125 for JNK (compare Fig. 7A and 7B). This difference suggests that pathways leading to LF gene activation by LPS and dsRNA both overlap but are unique. In addition, among the multiple pathways that induce LF expression by innate stimuli, only the NF- κ B pathway was shared by TNF- α induction in mouse mammary epithelial HC-11 cells.

Discussion

It is well established that LF inhibits bacterial and viral infections (review and references therein, [2]), however, the information on bacteria and virus induction of LF expression is limited and no mechanism of induction has been reported. In this study, we found that HC-11 cells responded to innate immune stimuli and produced LF to reduce their effects. In addition, we demonstrated that the PKC, MAPK and NF- κ B pathways were involved in the transactivation of LF gene expression.

LF is regulated by a complex net work of signaling pathways under the influence of hormones and during infection. To study the mechanism that regulates LF gene expression in epithelial cells faces a challenge, because the LF gene is permanently silenced in a majority of cancer cells [34-36]. Due to the lack of a suitable cell system for mechanistic study, most of the regulatory studies on LF were conducted in animals and in transiently transfected culture cell lines [7,17,37,38]. HC-11 is a normal mouse mammary gland cell line which retains the normal phenotype of mammary gland epithelial cells [29]. In addition, the LF gene is functional and can be regulated by hormones and an innate immune stimuli in this cell type (Fig. 1), thus offering an opportunity to examine the mechanism of innate stimuli that regulates LF expression in mammary gland epithelial cells.

LPS activates many genes involved in the inflammatory response through multiple signaling pathways in cell type and gene-specific manner [39-42]. One of the well characterized pathways for LPS is the NF- κ B signaling pathway in which the nuclear transcription factor, NF- κ B moves from the cytoplasm into the nucleus in response to LPS signaling, binds to the response element and induces target gene expression [24,25]. Several NF- κ B binding sites localized to the LPS-responsive regions of the bovine LF promoter were recently identified [7], although the presence of such an element has not yet been reported in other species including the mouse. Nonetheless, LPS-induced LF expression in HC-11 cells was effectively ablated by the IKK inhibitor (Fig. 6) which prevents nuclear translocation of NF- κ B suggesting that a NF- κ B binding element is present in the mouse LF promoter. Recently, Kruppel-like-factor 5 (KLF5), a member of the Kruppel-like factors family of transcription factors [43,44] which we have cloned and identified as a mouse LF mitogen response unit (MRU) binding protein [45], was thought to be an important mediator for LPS-induced proinflammatory response in intestinal epithelial cells [40]. Therefore, LPS could activate LF expression by enhancing the expression or activity of KLF5 in HC-11 cells. Additionally, LPS was known to activate the genes involved in inflammation through other signaling pathways such as PKC [42] and MAPK [41]. By using the inhibitors we showed that JNK and p38 MAPK, and PKC pathways are indeed involved in LPS induction of LF expression in HC-11 cells. Previously,

we found that transcription factors such as AP1, c-fos and c-jun, Sp1, Sp3 and KLF5 bind to the GT-box, AP1 and CRE binding elements of the mouse LF MRU and activate the transcriptional activity of the promoter [18,45,46]. These transcription factors could be recruited to mediate the MAPK and PKC signals activated by LPS exposure and stimulate the transcriptional activity of the LF gene.

dsRNA, another example of innate immune stimuli, also induces LF expression in HC-11 cells in a time- and dose-dependent manner. dsRNA is produced by most viruses during replication which recognizes the TLR3 on the cell membrane, upon binding and activation of the TLR3, dsRNA activates the NF κ B pathway [47]. It is also known that dsRNA is closely associated with the interferon (IFN) system in host defense [48] and innate cytokine induction in lung epithelial cells [22,27]. It is interesting to find that after initial recognition of the pathogen by the receptors on the cells membrane, the signaling pathways activated by the virus and bacteria merges and cross-talk between the pathways occurred [49,50]. In the HC-11 cells, dsRNA-induced LF expression was effectively abrogated by the same inhibitors that blocked LPS-induced LF (Fig. 6 and Fig. 7) suggesting that a potential cross-talk of the viral and bacterial activated pathways also exist in this cell type. Despite the similarities of LPS and dsRNA in response to inhibitors, differences were also found. PD 98059, a specific inhibitor of ERK [51], had little effect on LPS but reduced the dsRNA-induced LF expression by at least 50% (Fig. 7) implying that different factors were recruited in the pathways by dsRNA and LPS.

What is the significance of LF induction in response to innate stimuli? LF content was significantly increased in mammary gland during mastitis. The major source for LF came from the influx of polymorphonuclear neutrophils (PMN) which were recruited by the proinflammatory cytokines produced by the macrophages and the mammary gland epithelial cells [5]. The HC-11 mammary gland epithelial cell culture system confirms the *in vivo* observation that infection induces LF production in the mammary gland ([7] and Fig. 2). LF plays a major role in host defense against bacteria and viral via many different mechanisms in the extracellular environment [2]. Indeed, the majority of LPS and dsRNA-induced LF was found in the culture medium (Fig. 4) where it suppressed the activity of innate immune stimuli (Fig. 5). Interestingly, a small portion of the LF was found in the crude nuclear extract fraction of the cells by Western blotting. LF was reported to possess nuclear function by acting as a gene transcription factor by binding to specific DNA sequences [52] or interfering with cellular events leading to NF- κ B activation or suppression [53,54]. It is not clear whether LF in the HC-11 cells entered the nucleus and suppressed proinflammatory cytokine production by interfering with NF- κ B activation after LPS exposure. The possibility exists that the LF detected from the crude nuclear extract fraction was in fact associated with the cytoplasmic organelle. This assumption was based on the confocal image which indicated stronger staining around the perinuclear region, where the nuclear envelope connected with the endoplasmic reticulum of the cytoplasm (Fig. 4C). After LPS treatment, most of the cytosolic LF was released into the medium and the residual LF in the endoplasmic reticulum around the nuclei could either enter the nucleus or remain in the cytoplasm. In conclusion, the innate immune stimuli induce the expression of LF which in turn plays an inhibitory role in LPS and dsRNA induced TNF α production.

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Abbreviation used in this paper

LF, lactoferrin
 LPS, lipopolysaccharides
 dsRNA, double-stranded RNA
 PKC, protein kinase C
 PKA, protein kinase A
 MAPK, mitogen-activated protein kinase
 EGF, epidermal growth factor
 JNK, c-jun N-terminal kinase
 ERK, extracellular signal-regulated kinase
 NF- κ B, nuclear factor- κ B
 IL-1 β , interleukin 1 β

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Figure 1.

The mouse mammary epithelial HC-11 cells express LF and respond to estrogen stimulation. A. LF gene in HC-11 cells is estrogen responsive. Cells were seeded in the 60-mm dishes. The next day, they were treated with vehicle (0), 10 or 100 nM E2 or DES for 24 h. The mRNA level was detected by q-PCR and GAPDH used as an internal control. The data is presented as fold of induction $\pm$ S.E. B. Immuno-staining of LF in HC-11 cells. Cells were grown on Lab-Tek chamber slides overnight, fixed and then blocked the nonspecific sites with the serum blocking solution. The cells were stained with mouse LF antibody and then with Alexa Fluor 488 anti-FITC rabbit IgG antibody. The location of LF was visualized with a confocal microscopy using a 40×1.2 objective lens. The confocal images were acquired on the

microscopy at 512×512 resolutions over a 32 s exposure period. Image of the phase contrast is present at left and the immuno-staining at the right.

Figure 2.

Effect of LPS and dsRNA on LF expression in mouse mammary epithelial HC-11 cells. A. The dosage response curve for LPS. Cells were seeded in the 60-mm dishes. The next day, they were treated with vehicle (0), 1, 10, 100, 1000 or 10,000 ng/ml LPS for 24 h. Upper panel, total RNA was extracted and the LF mRNA level was determined by q-PCR. Lower panel, Western blotting analysis of the LF protein. Cells were treated as above and the whole cell lysates prepared in 1x Laemmli sample buffer. The LF protein was detected by Western blotting with anti-mouse LF serum (8345, 5,000x dilution) as described in the Materials and Methods. B. The time course for LPS. Upper panel, cells were treated with 100 ng/ml LPS for 0, 1.5, 3, 6, 12 or 24 h. The LF mRNA was determined by q-PCR. Lower panel, Western blot analysis of the LF protein. Cells were treated with 100 ng/ml LPS for 0, 24 or 48 h. The LF protein was detected by anti-mouse LF serum. C. The dosage response curve for dsRNA. Cells were seeded in the 60-mm dishes. The next day, they were treated with vehicle (0), 1, 5, 10 or 20 µg/ml dsRNA for 24 h. D. The time course for dsRNA. Cells were treated with 10 µg/ml dsRNA for 0, 1.5, 3, 6, 12 or 24 h. Total RNA was extracted and the LF mRNA level was determined by q-PCR. GAPDH mRNA or protein was used as control in either q-PCR or Western blotting in all experiments. The experiments were repeated three times and the results are presented as fold of induction $\pm$ S.E.

Figure 3.

Induction of proinflammatory cytokine expression by LPS and dsRNA in mouse mammary epithelial HC-11 cells. A. LPS induction of LF, TNF α , IL-1 β and IL-6. Gel picture of RT-PCR product. B. LPS induction of TNF α expression was determined by q-PCR. C. LPS induction of IL-1 β expression was determined by q-PCR. D. dsRNA induction of TNF α expression was determined by q-PCR. The same experiment was repeated three time and the samples were analyzed by q-PCR as described in the Materials and Methods. GAPDH was used as a control. The results are presented as fold of induction $\pm$ S.E.

Figure 4.

Cellular distribution of LF in mouse mammary epithelial HC-11 cells after LPS and dsRNA exposure. Cells were seeded in the 60-mm dishes and cultured in overnight before treatment. A. Detection of LF in the culture medium by Western analysis. Left panel, cells were treated with vehicle (0), 100 and 1,000 ng/ml of LPS for 24 h. Ten μl of culture medium (1/200), medium alone (negative control) and 100 ng of purified mouse LF (positive control) were analyzed in 10% PAGE gel and the presence of LF was detected by Western blotting with the anti-mouse LF serum (1:5,000x dilutions). Right panel, cells were treated with vehicle (0), 5 and 10 μg/ml dsRNA for 24 h. Ten μl of dsRNA treated culture medium, medium alone and 10 ng of purified mouse LF protein were analyzed by Western blotting. B. Detection of LF in cytosol and nuclear fraction of the HC-11 cells by Western blotting. Cells were treated with vehicle (0), 10, 100 and 1,000 ng/ml LPS for 24 h. The cells were fractionated by using the BD TransFactor Extraction Kits as described in the Materials and Methods section. 1/10 of the cytosolic or nuclear fraction was used for the analyses. GAPDH was used as a control. C. Detection of cellular distribution of LF by confocal microscopy. Cells were exposed to 100 ng/ml LPS for 4 h and the location of LF were examined by confocal microscopy as described in Materials and Methods. Left panel: phase contrast, Right panel: Immuno-staining of LF.

Figure 5.

LF blocked TNF- α expression in LPS and dsRNA exposed mouse epithelial HC-11 cells. A. LPS-induced LF blocked LPS's function. The LF containing conditioned medium (CM) was prepared from the LPS pre-exposed HC-11 cells as described in Materials and Methods section. Cells were maintained in the 10% FBS medium after seeding for overnight. For the experiments, the medium were changed into the normal FBS-free medium or the conditioned medium (also FBS-free) and then treated with 10 ng/ml LPS for 0, 0.5, 1, 2 or 3 h. Total RNA was extracted and the TNF α mRNA level was determined by q-PCR. GAPDH was used as a housekeeping gene. The experiments were repeated three times and results are presented as fold of induction $\pm$ S.E. B. Effect of exogenous mouse LF protein on the LPS induction of TNF α mRNA expression. Cells were seeded in the 60-mm dishes and cultured in 3 ml of 10% FBS medium overnight. After changing into the FBS-free medium, cells were pretreated with mouse LF protein (0-200 ng/ml) for 1 h and then treated with 10 ng/ml LPS for additional 2 h. The detection of TNF α mRNA by q-PCR was described as above. C. LF blocked the TNF- α induction by dsRNA. Cells were treated with vehicle (0) or 1 μ g/ml of dsRNA together with various concentrations of LF (0, 1, 5 and 10 μ g) for 2 h. The total RNA was extracted and the TNF- α mRNA was determined by q-PCR. The experiment was repeated three times. The result is presented as fold of induction $\pm$ S.E.

A

B **Figure 6.**

Effect of PKA, PKC and NF κ B signaling pathway inhibitors on LPS and dsRNA-induced LF expression in mouse mammary epithelial HC-11 cells. A. Effect on the LPS induction of LF (left panel) and TNF α (right panel) mRNA expression. Lower panel, Western blotting analysis to examine the effect of PKC and NF- κ B inhibitors on the LF protein production. Cells were seeded in the 60-mm dishes overnight, pretreated with 1 mM Calphostin C (PKC inhibitor) or 20 μ M 8-Bromoadenosine-cAMP, Rp-isomer (PKA inhibitor) or 5 μ M IKK inhibitor VII (NF- κ B inhibitor) for 1 h and then 100 ng/ml LPS was added for additional 3 h for mRNA level and 24 h for protein level determinations. B. Effect of inhibitors on the dsRNA induction of LF mRNA. Lower panel, Western blotting. Cells were seeded and pre-treated with inhibitors as in A and then 10 μ g/ml dsRNA was added for additional 3 h for mRNA level and 24 h for

protein level determinations. The total RNA was extracted and the mRNA levels were determined by q-PCR as described in Materials and Methods. GAPDH was used as a housekeeping gene. The experiment was repeated three times. The results are presented and fold of induction $\pm$ S.E.

Figure 7.

Effects of MAPK inhibitors on LPS and dsRNA-induced LF mRNA expressions in mouse mammary epithelial HC-11 cells. A. Effect on LPS induction. B. Effect on dsRNA induction. Cells were pre-treated with 50 μ M PD 98059 (ERK inhibitor), 20 μ M SB 203580 (p38 MAP kinase inhibitor) or 50 μ M SP 600125 (JNK inhibitor) for 1 h and then 100 ng/ml LPS or 10 μ g/ml dsRNA was added for additional 3 h. The LF mRNA levels were determined by q-PCR. Experiments were repeated three times and the results are presented as fold of induction $\pm$ S.E.