Relaxin Binds to and Elicits a Response from Cells of the Human Monocytic Cell Line, THP-1*

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Relaxin is a 6-kDa polypeptide with basic tertiary structural fold nearly identical to that of insulin (1). However, analysis of chemically synthesized relaxin derivatives has shown that the receptor-binding residues of the two proteins are discrete (2) and, although information is very limited (3, 4), the intracellular signaling events triggered when relaxin binds its receptor appear to be different from those triggered by insulin receptor binding. Consequently, it is not surprising that the physiological functions of relaxin are quite distinct from those of insulin and the other members of the insulin superfamily. Discovered in the 1920s, relaxin has been classically thought of as a “hormone of pregnancy” since it is present in the circulation at increased levels during pregnancy, when its synthesis is primarily directed by the corpus luteum. It serves to promote implantation of the blastocyst in the endometrium and maintenance of myometrial quiescence (5). In addition, relaxin and separation of the pelvic symphysis, and also acts to maintain quiescence of the myometrium (5). In this report, we provide evidence for a novel target of relaxin, the human monocyte/macrophage cell line, THP-1. Relaxin bound with high affinity (Kd = 102 pM) to a specific receptor on THP-1 cells. Receptor density was low (~275 receptors/cell), but binding of relaxin triggered intracellular signaling events. Receptor density was not modulated by pretreatment with estrogen, progesterone, or a number of other agents known to induce differentiation of THP-1 cells. Cross-linking studies showed radiolabeled relaxin bound primarily to cell surface proteins with an apparent molecular mass >200 kDa. Other members of the insulin-like family of proteins (insulin, insulin-like growth factors I and II, and relaxin-like factor) were unable to displace the binding of relaxin to THP-1 cells, suggesting that a distinct receptor for relaxin exists on this monococyte/macrophage cell line. Cross-linking studies showed radiolabeled relaxin bound primarily to cell surface proteins with an apparent molecular mass >200 kDa. Other members of the insulin-like family of proteins (insulin, insulin-like growth factors I and II, and relaxin-like factor) were unable to displace the binding of relaxin to THP-1 cells, suggesting that a distinct receptor for relaxin exists on this monococyte/macrophage cell line.

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1 E. Rinderknecht, unpublished data.

The abbreviations used are: RLF, relaxin-like factor; IGF, insulin-like growth factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT, room temperature; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; DSS, disuccinimidyl suberate; IBMX, isobutylmethylxanthine.
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extended periods of time have been observed for other stimuli. Therefore, in the following three studies, THP-1 cells were first washed with 100 mM NaOH, and lysates were transferred to microcentrifuge tubes containing 1 ml of OptiPhase SuperMix liquid scintillation mixture (Wallac) for determination of radioactivity in a Microbeta Plus liquid scintillation counter (Wallac). Percent specific binding was calculated as (total binding − nonspecific binding) ÷ (total binding) × 100.

In experiments designed to measure binding constants, triplicate samples containing 1 × 10⁶ THP-1 cells each were incubated at 22 °C for 60 min with increasing amounts of [³²P]-relaxin (20–400 pm) in the presence or absence of 1.4 μM unlabeled relaxin. Percent specific binding was determined as described above. To account for cell loss during the experiment, the number of cells was determined in two parallel samples. The number of relaxin binding sites, their affinity for relaxin, and the scatchard plot of the data were determined using the LIGAND program.

Cross-linking of [³²P]-Relaxin to Receptors on THP-1 Cells—Cells (5 × 10⁵) were incubated for 45 min at RT, in duplicate, with 400 pm [³²P]-relaxin in the presence or absence of 400 μM unlabeled relaxin or 400 μM insulin. Cells were washed with 1 ml of ice-cold PBS and resuspended in 100 μl of PBS containing 1 μM 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride (EDC), 1 mM dithiothreitol, 0.1 M sodium bicarbonate, pH 9.6. Recombinant human relaxin (lot M3RD-211, Genentech) was used as standard. The instrument utilizes a silicon sensor to detect small changes in pH in the extracellular fluid surrounding cells placed in a sensor chamber was 37°C.

Assaying Relaxin-induced cAMP Production—Relaxin was analyzed for its ability to induce cAMP production in THP-1 cells following the procedure of Fei et al. (3) with the following modifications. Logarithmically growing cells were centrifuged, resuspended in Dulbecco's modified Eagle's medium/F-12 medium (1:1, v/v) supplemented with 10% fetal bovine serum, 24 mM HEPES, pH 7.4, and 2 mM t-glutamate, and plated at a density of 80,000 cells/well in a 96-well culture plate. Relaxin diluted in assay diluent (Dulbecco's modified Eagle's medium/F-12 medium, 0.1% bovine serum albumin, 0.01% polysorbate 80), was added to the cells both with and without 1 μM forskolin and 50 μM isobutylmethylxanthine (IBMX). Following incubation at 37°C for 30 min, the plate was briefly centrifuged, and the medium was removed. Cells were lysed with 0.1 M HCl for 30 min at 37°C. Aliquots of the lysates were neutralized with 0.1 M NaOH, and cAMP levels were measured using the cAMP Biotrak EIA system (Amersham Corp.) according to the instructions of the manufacturer. Using a CAMP standard curve fit to a four-parameter equation, absorbance values were equated to CAMP concentrations. To examine the specificity of the relaxin-induced CAMP response, relaxin was preincubated with a 10-fold molar excess of an anti-relaxin monoclonal antibody, mAb8, prior to adding it to the THP-1 cells. This antibody is known to neutralize the ability of relaxin to induce a CAMP response in endothelial cells (3).

Assaying Relaxin-induced Changes in Extracellular Acidification Rate—Relaxin was analyzed for its ability to alter the basal acidification rate of THP-1 cells using a Cytoassensor microphysiometer (Molecular Devices). The instrument utilizes a silicon sensor to detect small changes in pH in the extracellular fluid surrounding cells placed in a microvolume sensor chamber (23–25).

Cells were resuspended at a density of 1.3 × 10⁶ cells/ml in running buffer (low buffered, serum-free, bicarbonate-free RPMI 1640 medium (Molecular Devices) containing 1% endotoxin-free human serum albumin (Miles)). Approximately 1 × 10⁶ cells were then immobilized in an agarose cell entrapment medium (Molecular Devices) and sandwiched between two 3-μm polycarbonate membranes (at a capsule cup (Molecular Devices). When the capsule cups were placed into the sensor chambers, cells were held in very close proximity to pH-sensitive detectors. Running buffer was pumped across the cells at a rate of 100 μl/min except during 30-s intervals when the flow was stopped, and acidification of the running buffer in the sensor chamber was measured. Acidification of unlabeled relaxin was determined every 2 min. The temperature of the sensor chambers was 37°C.

The cells were allowed to equilibrate in the sensor chambers for 1 h prior to the start of the experiment, and basal acidification rates were monitored during this time. Cells were then exposed to 100 μM of the phosphodiesterase inhibitor 4-butoxy-4-methoxybenzyl-2-imidazolidinone (RO-20-1724; Biomol Research Labs., Inc.) for 30 min. Cells were

³ S. Pitchford, personal communication.

Preparation of [³²P]-Relaxin—Relaxin was phosphorylated essentially as described (19, 20) with the following modifications. 25 μg of human B33 relaxin were incubated at 31°C for 30 min with 25 μl of [³²P]ATP (sp. act. 6000 Ci/hr/mmol) and 500 μl of the catalytic subunit of cAMP-dependent protein kinase (Promega) (introduced initially and 15 min after the reaction was begun) in a 200-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl₂. The reaction was terminated by placing the mixture on ice, and the unincorporated label was removed via chromatography on a Sep-Pak C18 cartridge (Waters). Radioactive fractions eluted from the cartridge were pooled, and their combined volume was reduced to 2 ml in a speed vac. Phosphorylated relaxin was separated from unphosphorylated protein on a Poly CAT-A ion-exchange column (PolyLC, Inc.) as described (19, 20). Fractions (0.5 ml) were collected in presiliconized microcentrifuge tubes (Sorenson BioScience, Inc.) containing 1 ml (10 mg/ml) of bovine serum albumin (Sigma) to minimize binding of the labeled relaxin to the tubes. Fractions containing peak levels of radioactivity were pooled and reduced to one-tenth of their original volume in a speed vac. Residual acetonitrile was eliminated by two rounds of dialution with 5 volumes of distilled water and subsequent evaporation. The concentration of the final [³²P]-labeled relaxin was determined by enzyme-linked immunosorbent assay. Labeled relaxin was stored at 4°C in a presiliconized microcentrifuge tube and subsequent evaporation. The concentration of the final [³²P]-labeled relaxin was determined by enzyme-linked immunosorbent assay. Labeled relaxin was stored at 4°C in a presiliconized microcentrifuge tube and was transferred to give 1

Relaxin was analyzed for its ability to alter the basal acidification rate of THP-1 cells using a Cytoassensor microphysiometer (Molecular Devices). The instrument utilizes a silicon sensor to detect small changes in pH in the extracellular fluid surrounding cells placed in a capsule cup (Molecular Devices). When the capsule cups were placed into the sensor chambers, cells were held in very close proximity to pH-sensitive detectors. Running buffer was pumped across the cells at a rate of 100 μl/min except during 30-s intervals when the flow was stopped, and acidification of the running buffer in the sensor chamber was measured. Acidification of unlabeled relaxin was determined every 2 min. The temperature of the sensor chambers was 37°C.

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then exposed to running buffer containing both the phosphodiesterase inhibitor and various concentrations of relaxin or other agents (diluted in running buffer). To examine the specificity of the acidification response to relaxin, the protein was incubated for 30 min at room temperature with a 10-fold molar excess of a neutralizing anti-relaxin monoclonal antibody (mAb8) prior to adding it to the running buffer/phosphodiesterase mixture. Acidification rates were expressed as percentages of the basal rates prior to the addition of relaxin. The data presented (see Fig. 6A) were collected in separate experiments and are the mean ± S.E. values from 2–4 capsule cups.

RESULTS

Binding of 32P-Relaxin to a Human Monocyte Cell Line—Recombinant human relaxin, 32P-labeled with cAMP-dependent kinase as described (19), bound specifically to cells from the human monocytic line, THP-1. The time course of binding at RT (22 °C) is shown in Fig. 1. Equilibrium was reached within 60 min, and specific ligand binding approached 80%. Binding was saturable since further incubation (to 90 min) did not significantly increase the amount of 32P-relaxin that was bound. The displacement of labeled relaxin from THP-1 cells by a variety of proteins from the insulin-like family is shown in Fig. 2. Both the mature B29 form of relaxin and the extended B33 form used for labeling reproducibly displaced 60–80% of the total radioactive relaxin that was bound. In contrast, human insulin, IGF-I, IGF-II, and the newly described RLF (26, 27) each typically displaced less than 20% of the bound 32P-relaxin. The binding constants for 32P-relaxin binding to receptors on THP-1 cells were obtained from Scatchard analysis of data from equilibrium binding experiments (Fig. 3). These data indicated a single class of high affinity binding sites (K_d = 102 pM) with a density of approximately 275 receptors/cell.

Because steroid hormones appear to regulate relaxin binding to certain targets, the effects of estrogen and progesterone on binding to THP-1 cells were analyzed. As shown in Fig. 4, estrogen and progesterone do not appear to regulate the expression of relaxin receptors on THP-1 cells. Cells treated for 48 h with 1 nM β-estradiol and/or 200 nM progesterone showed essentially the same binding capacity for 32P-labeled relaxin as

![Fig. 1. Time course of 32P-relaxin binding.](image)

![Fig. 2. Binding of 32P-relaxin to THP-1 cells and displacement by various proteins.](image)

![Fig. 3. Scatchard analysis of 32P-relaxin binding to THP-1 cells.](image)

![Fig. 4. The effect of estrogen and progesterone treatment on 32P-relaxin binding.](image)
did untreated cells. In addition, a 24–72-h pretreatment of THP-1 cells with various agents including 1α,25-dihydroxyvitamin D₃ (10, 30, 100 nM), lipopolysaccharide (25 μg/ml), retinoic acid (10 nM), interferon-γ (100 units/ml, 500 units/ml), phorbol 12-myristate 13-acetate (50 ng/ml), dexamethasone (25 nM), transforming growth factor β (10 ng/ml), phytohemagglutinin (100 ng/ml), and granulocyte macrophage colony-stimulating factor (10 ng/ml) did not significantly increase relaxin binding to specific binding sites (data not shown).

Binding of ³²P-Relaxin to Other Hematopoietic Cell Lines—In addition to the THP-1 cell line, four other monocytic/macrophage lines, the human K562 and U937 and the murine J774A.1 and RAW264.7 lines, demonstrated specific relaxin binding. In these lines, specific binding accounted for 10–40% of the total radioligand that was bound. Thirteen other hematopoietic cell lines were examined for their ability to bind ³²P-relaxin. None of these lines (HEL 92.1.7, KG-1a, RS4;11, HL-60, P815, 10P12, MEG-01, Jurkat, EBV) showed significant specific relaxin binding.

Cross-linking of ³²P-Relaxin to Receptors on THP-1 Cells—Characterization of the THP-1 proteins that bind to relaxin was initiated by cross-linking the bound radioactive ligand to THP-1 cells and separating the complexes by SDS-PAGE under non-reducing (−β-mercaptoethanol) or reducing (+β-mercaptoethanol) conditions (Fig. 5). Two different cross-linking agents that differed in their reactivity and membrane permeability were employed. One EDC linked amine groups to nearby carbonyl groups and was membrane-impermeable. The other, DSS, was a homobifunctional cross-linker that joined adjacent amine groups and was membrane-permeable. Parallel binding experiments were also performed in which no cross-linking agent was added. Both cross-linkers revealed a >200-kDa species bound to the labeled relaxin that could be completely displaced by a 1000-fold molar excess of unlabeled relaxin but not with a 1000-fold molar excess of insulin. Equivalent amounts of IGF-I, IGF-II, and RLF were similarly unable to compete with the ³²P-relaxin for binding and cross-linking to this >200-kDa receptor (data not shown). Cross-linking in the presence of EDC also revealed an additional minor ~100-kDa species bound to the labeled relaxin. Binding and cross-linking of the labeled ligand to this protein was also displaced with excess unlabeled relaxin but not with excess insulin, IGF-I, IGF-II, or RLF (Fig. 5; data not shown).

Relaxin Binding Triggers a cAMP Response in THP-1 Cells—There is a dose-dependent increase in intracellular cAMP content observed following a 30-min incubation of THP-1 cells with increasing concentrations of relaxin (Fig. 6A). No such increase is observed when the cells are incubated with placebo. The response is similar to that seen in endometrial cells (3). In those cells, treatment with a phosphodiesterase inhibitor, IBMX, to block the breakdown of cAMP and a low concentration of forskolin to activate adenyl cyclase is required to observe a relaxin-induced increase in cAMP. The presence of both of these agents is required for maximal cAMP accumulation in response to relaxin in THP-1 cells as well (Fig. 6A); however, the relaxin response in their absence is greater than that seen in endometrial cells (data not shown). The cAMP response of THP-1 cells to relaxin is highly specific. Preincubation of relaxin with a 10-fold molar excess of an anti-relaxin monoclonal antibody, mAb8, completely inhibited the ability of relaxin to induce a cAMP response (Fig. 6B). Furthermore, incubation of THP-1 cells with identical molar concentrations of insulin, IGF-I, IGF-II, or RLF did not elicit a cAMP response.

Relaxin Binding Induces an Increase in Extracellular Acidification Rate by THP-1 cells—Interaction of a ligand with its cell surface receptor often results in a fluctuation in the basal rate at which the cell secretes acidic metabolites (e.g. CO₂, lactic acid) into the surrounding medium (23, 28). A silicon microphysiometer can be used to measure, in real time, these transient changes in living cells (24, 25). Observation of a perturbation in acidification rate, therefore, provides evidence for an intracellular signaling event transduced in response to binding of the ligand to its receptor. As shown in Fig. 7A, relaxin triggers a dose-dependent increase in the extracellular acidification rate in THP-1 cells. THP-1 cells were preincu-
Relaxin binds to a highly specific receptor/binding site on THP-1 cells. Other proteins in the insulin superfamily, including insulin, IGF-I, IGF-II, and RLF (also known as Leydig cell factor) are not capable of displacing labeled relaxin even when present in great (1000-fold) excess. Similar results have been reported for the binding of relaxin to endometrial cells and cardiomyocytes, as well as to various membrane and tissue preparations (4, 19, 20, 29). In contrast, insulin and IGF-I each have moderate affinity for the receptors of the other, and their receptors are closely related in both structure and function (30–32). Thus, the displacement data suggest that THP-1 cells bear relaxin-specific receptors that are distinct from insulin/IGF-type I and RLF receptors.

Relaxin appears to bind to a single class of binding sites on THP-1 cells. Similar findings have been reported from studies using cells and tissues from the brain, heart, and uterus (4, 20, 29). The affinity of relaxin for the THP-1 receptors \( K_D = 100 \) pM) is slightly higher than that reported for receptors on these other cell types. Dissociation constants for relaxin binding to crude membrane fractions and tissue sections from brain, heart, and uterus are generally 1–2 nM, and binding experiments using intact endometrial cells have given a \( K_D \) of 440 pM. Perhaps there are accessory proteins on THP-1 cells that contribute to their highly specific, high affinity relaxin binding. In all cells and tissues examined thus far, relaxin receptor abundance is very low, and THP-1 cells are no exception with 275 receptors/cell. Human endometrial cells have \(~1000\) receptors/cell (4), and rat atrial cardiomyocytes have \(~210\) receptors/cell.4 Despite low receptor abundance, however, it is clear that signal transduction does occur following ligand binding.

Relaxin both stimulates the production of an intracellular second messenger and produces a change in the extracellular environment of THP-1 cells within 10–30 min of receptor binding. The magnitude of the dose-dependent increase in cAMP is similar to that induced by relaxin binding to endometrial cells (3). The response is also similar to the endometrial cell response in that it is enhanced by agents that block cAMP breakdown (IBMX) or stimulate adenyl cyclase ( forskolin), but the THP-1 response is larger than that in endometrial cells in the absence of these compounds. Because the cAMP concentration changes induced by relaxin are enhanced by the presence of IBMX, it is likely that an increase in cAMP is associated with the release of acidic metabolites. Finally, recent studies show that, in addition to second messenger induction, relaxin also stimulates transcription of vascular endothelial growth factor, a cytokine capable of causing endothelial cell proliferation (33). This indicates that the relaxin receptors on the surface of THP-1 cells are functionally linked to the transcriptional machinery of these cells.

Experiments measuring relaxin binding to tissue sections indicate that endometrial relaxin receptors are up-regulated following estrogen priming in vivo, whereas heart and brain

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4 D. A. Parsell and J. Y. Mak, unpublished observations.
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Relaxin receptors are not (6, 7). We examined relaxin binding to THP-1 cells following estrogen, progesterone, and estrogen plus progesterone treatment. 32P-Relaxin binding was not altered in response to any of these pretreatments. Relaxin receptors on THP-1 cells are, therefore, similar to those on heart and brain tissue in this regard.

Cross-linking of 32P-relaxin to its receptor on THP-1 cells results primarily in a labeled complex that migrates with an apparent molecular mass of >200 kDa on SDS-PAGE. A minor species migrating at ~100 kDa was also detected when a membrane-impermeable cross-linker, EDC, was employed. There was very little change in the pattern of the labeled bands upon running the samples under reducing conditions. The slight increase in the intensity of the 100-kDa band could indicate that the receptor is composed of two ~100-kDa subunits, but the subtle nature of the difference in mobility between reducing and non-reducing conditions makes this difficult to conclude. It is possible that DSS, the membrane-permeable cross-linker, more efficiently cross-links the subunits to each other, preventing their separation on the reducing gel. Our studies are in good agreement with cross-linking experiments conducted with relaxin bound to human endometrial cells since these cells also show an ~200-kDa receptor (4). A 100-kDa band was not seen in those experiments, but DSS was the only cross-linking agent employed.

It is not yet clear whether monocyte/macrophage populations, which are relaxin targets, exist in vivo. The hematopoetic cell lines that exhibited relaxin binding (THP-1, K562, U937, and RAW264.7) are relatively undifferentiated, as judged by expression of immature monocyte markers. Maturation of these lines using a number of cytokines and agents known to cause monocyte differentiation either did not alter relaxin binding or decreased it. We therefore believe that a candidate relaxin target may be a fairly immature monocyte subpopulation. Consistent with this hypothesis, we have not observed binding of labeled relaxin to human peripheral blood mononuclear cells, either to the CD14+ or CD14− depleted populations (data not shown). We have also not observed relaxin binding to rodent tissue macrophages that are resident in the lung or peritoneum (data not shown). However, in preliminary experiments, we have observed specific relaxin binding to cells in rodent bone marrow. We are currently attempting to identify the relaxin-binding population in the marrow and to characterize the functional consequences of this binding. In summary, we have described, for the first time, binding of human relaxin to a cell type of monocyte/macrophage lineage. Binding results in second messenger production and rapid metabolic changes in the cell. These data may indicate a novel role for relaxin in macrophage biology.

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