Nickel Is a Specific Inhibitor for the Binding of Activated α_2-Macroglobulin to the Low Density Lipoprotein Receptor−Related Protein/α_2-Macroglobulin Receptor†

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ABSTRACT: The low density receptor-related protein/α_2-macroglobulin receptor (LRP/α_2-MR) binds to several ligands involved in lipoprotein and protease clearance. The receptor-associated protein (RAP) inhibits the binding of all known ligands. We studied the inhibition by Ni^{2+} of the binding of different ligands to cells and to the purified LRP/α_2-MR. Ni^{2+} inhibited all of the specific binding of radiolabeled methylamine-activated α_2-macroglobulin (^{125}I-α_2-M*) to rabbit aortic smooth muscle cells (SMC), rat hepatoma Fu5AH, and mouse fibroblast L cells. Ni^{2+} also inhibited the binding of trypsin-activated α_2-macroglobulin to SMC but did not affect the binding of RAP, *Pseudomonas* exotoxin A, or low-density lipoproteins. The inhibition of α_2-M* binding by Ni^{2+} was not due to its interaction with α_2-M*. Preincubation of SMC with Ni^{2+} followed by ligand binding suggested that Ni^{2+} binds to cell-surface molecules and inhibits the binding of α_2-M* but does not affect RAP binding. Most of the binding of α_2-M* to SMC was due to its binding to the LRP/α_2-MR, as opposed to the recently described signaling receptor, as demonstrated by the inhibition of this binding by the RAP. Moreover, the inhibition of α_2-M* binding to the LRP/α_2-MR by Ni^{2+} was demonstrated using purified receptor immobilized on microtiter plates. Two to three molecules of $^{63}Ni^{2+}$ bound to the immobilized receptor with equal affinity but not to α_2-M*. The specific binding of α_2-M* to the immobilized receptor was inhibited in the presence of nickel. Furthermore, preincubation of the immobilized LRP/α_2-MR with Ni^{2+} inhibited the binding of α_2-M* but did not inhibit RAP or exotoxin A binding. These data suggest that Ni^{2+} is a site-specific inhibitor for the α_2-M* binding site present on the LRP/α_2-MR. Nickel may be a useful tool for investigating different ligand binding domains of the LRP/α_2-MR.

Native α_2-macroglobulin molecule is a general protease inhibitor that interacts with all four classes of proteases [for reviews, see Sottrup-Jensen (1989), Gonias (1992), and Chu and Pizzo (1994)]. After its interaction with proteases, α_2-macroglobulin undergoes a conformational change, resulting in the entrapment of proteases and exposure of a receptor-binding site at its C-terminus; in this state it is called activated α_2-macroglobulin (α_2-M*).[1] The α_2-M* is cleared very rapidly from plasma by receptor-mediated endocytosis [for reviews, see Sottrup-Jensen (1989), Gonias (1992), and Chu and Pizzo (1994)]. The major receptor involved in the clearance of α_2-M* is the low density receptor−related protein/α_2-macroglobulin receptor (LRP/α_2-MR) [for reviews, see Brown et al. (1991), Herz (1993), Krieger and Herz (1994), Moestrup (1994), and Strickland et al. (1995)]. Recently, evidence has been presented for the presence in macrophages of a signaling receptor, separate from the LRP/α_2-MR, that binds α_2-M* and increases intracellular calcium (Misra et al., 1994a,b).

The LRP/α_2-MR is a multifunctional receptor that recognizes several ligands involved in the metabolism of proteases and lipoproteins [for reviews, see Brown et al. (1991), Herz (1993), Krieger and Herz (1994), Moestrup (1994), and Strickland et al. (1995)]. It consists of two chains, 515 and 85 kDa (Jensen et al., 1989; Herz et al., 1990; Kristensen et al., 1990; Strickland et al., 1990). All the ligands described so far bind to the 515-kDa polypeptide (Brown et al., 1991; Herz et al., 1991; Moestrup & Gliemann, 1991; Herz, 1993; Krieger & Herz, 1994; Moestrup, 1994; Godyna et al., 1995; Kounnas et al., 1995). Different ligands that bind to the receptor usually do not cross-compete very well with each other, suggesting the presence of distinct, but partially overlapping, binding sites on the receptor molecule. Based on the identified ligands, a minimum of five binding sites may exist on this receptor molecule [for reviews, see Brown et al. (1991), Herz (1993), Krieger & Herz (1994), Moestrup (1994), Strickland et al. (1995)]: (1) an α_2-M* binding site (which is not competed by other ligands); (2) an apoE/β-VLDL and lactoferrin binding site; (3) a lipoprotein lipase/β-VLDL binding site [a partial competition for this site has been observed by α_2-M* (Chappell et al., 1992)]; (4) a plasminogen activator inhibitor-1 binding site; and (5) an urokinase−plasminogen activator binding site. Urokinase−plasminogen activator/plasminogen activator inhibitor-1 com-

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[1] Abbreviations: α_2-M*, methylamine-activated α_2-macroglobulin; SMC, rabbit aortic smooth muscle cells; LRP/α_2-MR, low-density receptor-related protein/α_2-macroglobulin receptor; RAP, receptor-associated protein; apoE/β-VLDL, apolipoprotein E/β-very low density lipoprotein.

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plexes bind LRP/α_2-MR with high affinity presumably because of the combined interactions of these molecules to the receptor (Moestrup, 1994). In contrast to these ligands, the receptor-associated protein (RAP) inhibits the binding of all the ligands to the receptor (Herz et al., 1991; Moestrup & Glieman, 1991; Willnow et al., 1992; Herz, 1993). The RAP copurifies with the receptor (Jensen et al., 1989; Kristensen et al., 1990; Strickland et al., 1990), and binds to two (Williams et al., 1992) or several (Iadonato et al., 1993) equivalent binding sites. It has been suggested that the binding of RAP to specific sites results in the inhibition of the binding of different ligands to the receptor; i.e., it acts as a competitive inhibitor. It has also been speculated that RAP may inhibit the binding of different ligands by an allosteric mechanism (Herz, 1993). Warshawsky et al. studied the binding of different regions of RAP that inhibit the binding of α_2-M* and tissue type-plasminogen activator to the receptor, and suggested that "RAP inhibits ligand binding indirectly, for example by steric hindrance" (Warshawsky et al., 1994).

The binding domains for different ligands present on the LRP/α_2-MR have not been identified. Two different approaches have been made toward identifying the different ligand-binding domains. First, binding of ligands to different receptor fragments was studied. A fragment of 776−1399 amino acids recognized rat α_1-macroglobulin light chain, urokinase−plasminogen activator inhibitor type-1 complex, and RAP (Moestrup et al., 1993). Second, mini-receptor constructs were overexpressed in Chinese hamster ovary cells, and their binding to different ligands was studied (Willnow et al., 1994). Two different ligand binding domains were identified: one domain of amino acids 836−2501 binds to RAP, tissue-type plasminogen activator, and tissue-type plasminogen activator/plasminogen activator inhibitor-1 complexes; and the second domain of amino acids 3316−4167 binds to RAP (Willnow et al., 1994). Since the 776−1399 fragment bound rat α_1-macroglobulin light chain whereas the 836−2501 fragment did not bind α_2-M*, it was suggested (Willnow et al., 1994) that the α_2-M* binding site may require amino acids 776−836.

The LRP/α_2-MR requires extracellular calcium for its binding activity Herz et al. 1988, 1991; Kowal et al., 1989; Moestrup & Gliemann, 1989; Nykjaer et al., 1992; Orth et al., 1992; Williams et al., 1992). The receptor has at least eight high-affinity binding sites for the Ca^{+2} that can be substituted by Sr^{+2} or Ba^{+2} but not by Pb^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, or La^{3+} (Moestrup et al., 1990). Because binding of ligands to the receptor has been demonstrated to increase intracellular calcium concentrations (Misra et al., 1994b), we studied the effect of calcium channel blockers on receptor activity. To our surprise, Ni^{2+}, an inorganic calcium channel blocker, inhibited the binding of α_2-M* to the LRP/α_2-MR. Ni^{2+} and other group VIII transition metals have been shown to block dihydropyridine-sensitive Ca^{2+} channels (Nachsen, 1984; Winegar et al., 1991). Nickel has been considered an essential ultra-trace element for mammals with human requirements of less than 100 μg/day (Nielsen, 1991). It is a component of urease in plants and methyl-CoM reductase in methanogenic bacteria, and probably in propionyl-CoA carboxylase in mammals (Friedmann et al., 1990; Nielsen, 1991). Deprivation of Ni^{2+} has been associated with depressed growth and reproductive function in rats, goats, and minipigs. However, toxicity associated with Ni^{2+} may be more important for human health. Nickel is a known carcinogen, teratogen, and contact allergen (Anonymous, 1990; Nordlind, 1990; Costa, 1991; Sunderman, 1993), although its mode of action is poorly understood.

MATERIALS AND METHODS

Materials. Native α_2-macroglobulin purified from human plasma, trypsin-activated α_2-macroglobulin, and purified LRP/α_2-MR (Strickland et al., 1990) were generously provided by Drs. M. Kounnas and D. Strickland of the American Red Cross, Rockville, MD. The bacterial plasmid expressing the RAP (Herz et al., 1991) was a generous gift of Dr. Herz of the University of Texas Southwestern Medical Centr, Dallas, TX. *Pseudomonas* exotoxin A, divalent cations, and albumins were obtained from Sigma, St. Louis, MO. Human LDL was obtained from lipoprotein core of the Department of Biochemistry and used within three weeks of preparation. Cell culture media were obtained from Mediatech, Washington, DC.

Smooth Muscle Cells. Rabbit thoracic aortic smooth muscle cells (SMC) were prepared by the enzyme dispersion method and cultured in minimum essential medium containing 10% fetal bovine serum as described earlier (Ross, 1971; Gleason et al., 1993). For experiments, SMC from 2−15 passages were plated in 24-well plates and used after 1 week. Cultures were confluent at the time of the experiment.

Cell Binding Experiments. Human plasma α_2-macroglobulin was activated by incubating with methylamine (200 mM, 2 h, 22 °C) and purified by passing through a PD10 column (Biorad, Richmond, CA). The α_2-M* was iodinated as described earlier (Hussain et al., 1991; Kancha et al., 1994). All binding experiments were performed at 4 °C as described previously (Hussain et al., 1991; Kancha et al., 1994). For these experiments, cells were incubated in serum-free medium for 30 min at 37 °C, and in minimum essential medium containing 15 mM HEPES for 1 h at 4 °C. Cells were washed and incubated in the same medium supplemented with 5 mM $CaCl_2$, 5% bovine serum albumin, and radiolabeled ligands, in duplicate, for 4 h at 4 °C. At the end of the incubation, cells were washed three times with phosphate-buffered saline containing 5 mM $CaCl_2$ (Isaacs et al., 1988), collected in 0.1 N NaOH, and counted in a gamma counter (Packard, Meriden, CT). ^{125}I-Labeled ligands used for total binding were α_2-M* (0.725 μg/mL; 1 nM), trypsin-activated α_2-macroglobulin (0.725 μg/mL; 1 nM), RAP (0.5 μg/mL; 13 nM), *Pseudomonas* exotoxin A (2 μg/mL), and human low-density lipoprotein (2 μg protein/mL). To determine nonspecific binding, 150-fold excess unlabeled ligands were included. The total and nonspecific binding were determined in duplicate, and specific binding was deduced by subtraction. Cell protein was measured by the method of Bradford (Bradford, 1976) using a Coomassie plus reagent (Pierce, Rockford, IL) and bovine serum albumin as standard.

Receptor Binding Experiments. The purified LRP/α_2-MR (5 μg/mL in 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) was added to microtiter wells (100 μL/well) and incubated overnight at 4 °C. The wells were then blocked with 100 μL of 10 mg of dog serum albumin/mL of 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 for 1 h at room temperature, washed and then incubated with different

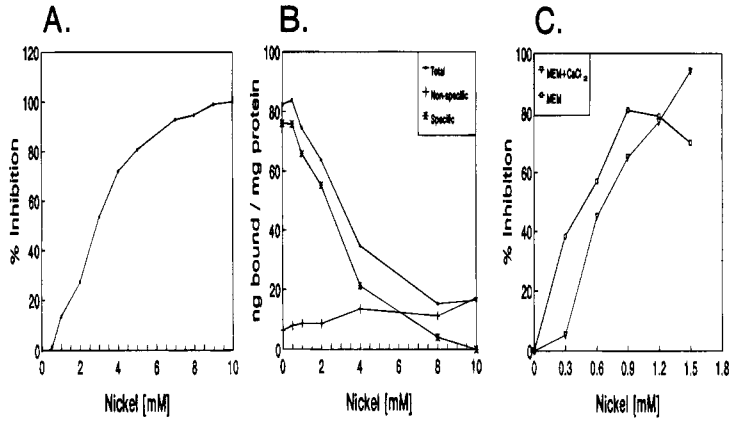

FIGURE 1: Effect of different concentrations of nickel on the binding of α_2-M* to smooth muscle cells. SMC were incubated with ^{125}I-α_2-M* (1 nM) in the presence and absence of unlabeled α_2-M* and different indicated concentrations of Ni^{2+} for 4 h at 4 °C as described in Materials and Methods. Cells were washed, extracted in 0.1 N NaOH, and counted. (A) Inhibition of receptor activity by increasing concentrations of Ni^{2+}. (B) Effect of Ni^{2+} on total, specific, and nonspecific binding. Nonspecific binding was determined in the presence of 150-fold excess unlabeled α_2-M*. (C) Inhibition of receptor activity by increasing concentrations of Ni^{2+} in the absence of albumin with or without addded $CaCl_2$ (5 mM). Each point is an average of duplicate determinations.

concentrations of nickel chloride (100 mM stock was prepared in 50 mM Tris, 150 mM NaCl, pH 8.0) in the presence of radiolabeled α_2-M* for 18 h at 4 °C. Plates were washed and counted. For preincubation experiments, receptor was first preincubated for 6−8 h at 4 °C with different concentrations of nickel and then incubated overnight at 4 °C with different ligands in the absence of Ni^{2+}. After the incubation, wells were washed twice and counted in a gamma counter.

Nickel-63 Binding to Purified LRP/α_2-MR. Receptor was immobilized and nonspecific sites were blocked using pig serum albumin (1 mg/well) as described above. After two washes, immobilized receptor was incubated with different concentrations of $^{63}NiCl_2$ (specific activity, 12.3 mCi/mg; DuPont, Wilmington, DE) in 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 for 18 h at 4 °C. Wells were washed twice, placed in individual scintillation vials, incubated with 100 μL of 0.1 N NaOH for 10 min, and counted in the presence of 3 mL of scintillation cocktail (ScintiVerse, Fisher Scientific, NJ). The binding of nickel was analyzed by nonlinear regression and Scatchard analyses using Inplot (GraphPad, San Diego, CA).

The amount of proteins bound to microtiter plates was quantified using radiolabeled proteins. Proteins (200 μg) were incubated with Iodogen (1 mg) and ^{125}I (0.2 μCi) for 5 min (Hussain et al., 1991; Kancha et al., 1994). Radiolabeled proteins were purified using PD10 column. Known amounts of protein were incubated in microtiter plates as described for studying the binding of $^{63}Ni^{2+}$ to the immobilized purified receptor.

RESULTS

Effect of Nickel on LRP/α_2-MR Activity. We studied the effect of different Ca^{+2}-channel blockers on the activity of LRP/α_2-MR in SMC (Table 1). Cells were incubated with the blockers for 48 h at 37 °C, washed, and used for the binding of α_2-M* at 4 °C. Only Ni^{2+}, an inorganic calcium channel blocker, inhibited the binding of α_2-M* by 90%. Furthermore, addition of Ni^{2+} to chilled cells during binding experiments at 4 °C was sufficient to inhibit the binding of α_2-M* to SMC (Figure 1). This suggested that the effect of Ni^{2+} was not dependent on changes in mRNA or protein levels.

Table 1: Effect of Calcium Channel Blockers on the Cell-Surface Activity of LRP/α_2-MR in Normal Aortic Smooth Muscle Cells

treatment[a]	n[b]	specific binding (ng bound/mg of cell protein)
control	13	62.1 ± 19.8
amlodipine	5	66.2 ± 25.2
nifedipine	2	69.0, 97.9
diltiazen	2	59.0, 74.3
nickel	4	6.4 ± 3.7[c]

[a] Smooth muscle cells were incubated with different calcium channel blockers for 48 h at 37 °C and used for α_2-M* binding experiments at 4 °C as described in Materials and Methods. The concentrations used were as follows: amlodipine (1 nM), nifedipine (1 nM), diltiazen (1 nM), and nickel chloride (10 mM). [b] Number of different experiments. Each experiment was performed in duplicate. [c] Mean ± SD, $p < .001$.

Nickel did not inhibit the binding of α_2-M* to SMC at 0.5 mM in the presence of bovine serum albumin (Figure 1A). However, a progressive increase in the inhibition of binding of α_2-M* to SMC was observed with increasing concentrations of Ni^{2+}. Fifty percent inhibition was observed at 3 mM Ni^{2+} (Figure 1A), whereas complete inhibition was observed at 9 mM. The inhibition of α_2-M* binding by Ni^{2+} was due to decreased specific binding of α_2-M* to SMC (Figure 1B). The concentration of Ni^{2+} required for the inhibition was affected by the presence of bovine serum albumin in the binding medium because it has one high-affinity binding site and several low-affinity binding sites for Ni^{2+} (Predki et al., 1992). Thus, binding experiments were repeated in the absence of albumin (Figure 1C), and, in this case, the amount of Ni^{2+} required for 50% inhibition of the specific binding of α_2-M* in minimum essential medium supplemented with 5 mM $CaCl_2$ was 0.7 mM. Next, binding experiments were performed in minimum essential medium (which contains 1.8 mM $CaCl_2$) without the addition of extra calcium and albumin. In the absence of added $CaCl_2$, 0.5 mM Ni^{2+} inhibited 50% of the specific binding to SMC (Figure 1C), suggesting that 4- to 6-fold lower levels of Ni^{2+} are required for 50% inhibition of α_2-M* binding to SMC when binding experiments are performed without added albumin and $CaCl_2$.

Consideration was given to the possibility that Ni^{2+} inhibited the α_2-M* binding by altering the pH of the binding medium. Addition of nickel to medium decreased the pH

Table 2: Effect on pH on the Binding of Activated ^{125}I-α_2-Macroglubulin to Smooth Muscle Cells

conditions[a]	pH	binding (μg of α_2-macroglobulin/mg of cell protein) total	nonspecific	specific	% inhibition[b]
medium	7.4	40.6 ± 8.2[c]	2.0 ± 0.2	38.6 ± 7.9	0
medium + Ni^{2+}	7.4	10.7 ± 0.5	6.0 ± 1.7	4.7 ± 1.3	88
medium	6.8	51.9 ± 7.1	1.6 ± 0.4	50.3 ± 6.7	0
medium + Ni^{2+}	6.8	25.5 ± 0.6	13.4 ± 0.1	12.2 ± 0.6	76

[a] The pH of the binding medium with or without nickel (10 mM) was 6.8 and 7.4, respectively. Thus, binding media with or without nickel were adjusted to pHs as indicated; cells were incubated in these media with ^{125}I-labeled α_2-M* for 4 h at 4 °C as described in Materials and Methods, washed, extracted in 0.1 N NaOH, and counted. [b] Inhibition (%) of specific binding of α_2-M* due to the addition of nickel chloride (10 mM) during ligand binding experiments. [c] Mean ± SD, n = 3.

from 7.4 to 6.8. Thus, binding experiments were performed at both pHs; binding at these pHs did not significantly differ from each other, suggesting that lowering of pH to 6.8 itself does not result in decreased binding of α_2-M* to SMC (Table 2). Next, we studied the effect of inclusion of nickel at two different pHs. The presence of nickel at both pHs significantly inhibited the specific binding of α_2-M* to SMC (Table 2), suggesting that inhibition by nickel was independent of pH. Thus, we conclude that the inhibition of α_2-M* binding was due not to changes in pH but to the presence of Ni^{2+} in the binding medium.

The effect of Ni^{2+} was not limited to SMC only. It inhibited all of the specific binding of α_2-M* to rat hepatoma Fu5AH, and mouse fibroblast L cells (Table 3). These studies suggest that Ni^{2+} is a specific inhibitor for α_2-M* binding in a variety of cells.

Effect of Nickel on the Binding of Different Ligands to SMC. Nickel inhibited the binding of both methylamine- and trypsin-activated α_2-macroglobulin (Table 4). It inhibited the binding of neither *Pseudomonas* exotoxin A (known ligand of the LRP/α_2-MR) nor low-density lipoproteins to SMC. In fact, a 2-fold increased binding of these ligands was observed. These studies suggest that Ni^{2+} is a specific inhibitor for the binding of activated α_2-macroglobulins to SMC.

Next, we studied the effect of Ni^{2+} on RAP binding (Figure 2). 4 mM Nickel inhibited ^{125}I-α_2-M* binding by 75% but did not affect RAP binding (Figure 2A) suggesting that Ni^{2+}, at concentrations that significantly inhibit α_2-M* binding, does not affect RAP binding to SMC. At higher concentrations (6 mM and above), however, Ni^{2+} affected both specific and nonspecific binding of RAP to SMC (Figure 2B). Agarose gel electrophoresis demonstrated that RAP had increased positive charge (data not shown) in the presence of 10 mM Ni^{2+} indicating a direct interaction between RAP and Ni^{2+} that may be responsible for the observed changes in nonspecific and specific binding of RAP to SMC. A direct evidence for the interaction between Ni^{2+} and RAP in the absence of cells was obtained by incubating them in cell culture plates without cells. Higher concentrations of Ni^{2+} increased the binding of RAP to plastic, indicating that Ni^{2+} interacts with RAP (data not shown). Thus, to examine the effect of Ni^{2+} on RAP binding, we adopted the preincubation protocol described below.

Effect of Preincubation of SMC with Nickel on the Binding of α_2-M. The studies described so far, however, do not rule

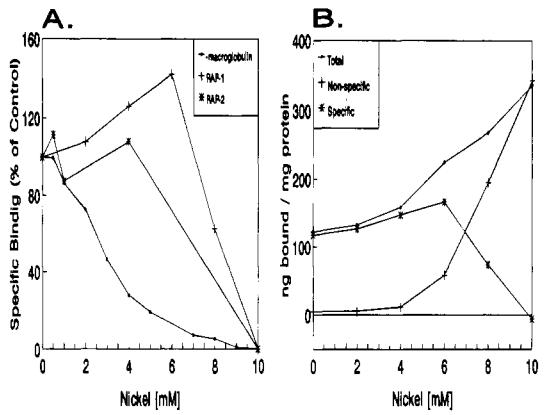

FIGURE 2: Effect of nickel on the binding of the receptor-associated protein to smooth muscle cells. (A) SMC were incubated in duplicate with radiolabeled α_2-M* or RAP as described in Materials and Methods, in the presence of different concentrations of Ni^{2+}. Effect on specific binding of α_2-M* is shown for comparison (data is from Figure 1). Results from two different experiments displaying the effect of Ni^{2+} on RAP binding are shown. (B) Effect of Ni^{2+} on the total, specific, and nonspecific binding of RAP to SMC.

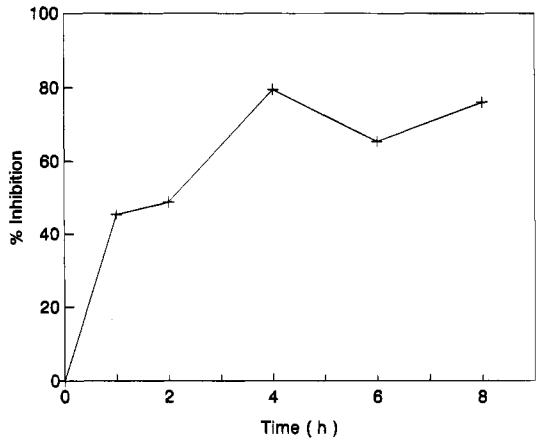

FIGURE 3: Effect of preincubation of smooth muscle cells with nickel on the binding of α_2-M*. SMC were preincubated with 10 mM Ni^{2+} in minimum essential medium containining 10% fetal bovine serum for indicated times at 4 °C. Cells were washed once with serum-free minimum essential medium containing HEPES and incubated with ^{125}I-labeled α_2-M* for 4 h in the binding medium as described in Materials and Methods.

out the possibility that Ni^{2+} interacts with α_2-M*, as it does with RAP, and decreases its binding to the receptors present on the cell-surface of SMC. Evidence for the interaction between Ni^{2+} and cell-surface receptors was obtained by preincubating SMC with Ni^{2+} (10 mM) for different times and then performing the binding experiments (Figure 3). Preincubation for 1−2 h resulted in approximately 50% inhibition of the binding of α_2-M* to the receptor and 70−80% inhibition was observed at 4−8 h of preincubation. These studies suggest that Ni^{2+} binds to SMC and inhibits the binding of α_2-M* to its receptor. Next, we studied the effect of preincubation of SMC with Ni^{2+} on RAP binding. Preincubation of SMC with Ni^{2+} for 4 h at 4 °C resulted in 90% inhibition of specific α_2-M* binding, but under similar conditions no inhibition of the specific binding of RAP was observed. In fact, a 25% increase in specific binding was observed. Moreover, no increase in nonspecific binding of RAP to SMC was observed under these conditions. These studies suggest that Ni^{2+} interacts with the cell-surface receptor and inhibits the binding of α_2-M* but does not affect

Table 3: Effect of Nickel on the Binding of Activated ^{125}I-Labeled α_2-Macroglobulin to Different Cells

cells[a]	binding (μg of α_2-macroglobulin/mg of cell protein)			% inhibition[b]
	total	nonspecific	specific	
rabbit aortic smooth muscle cells				
control	75.3 ± 16.0[c]	11.2 ± 4.9	64.1 ± 16.1	
Ni^{2+}	17.6 ± 3.0	12.1 ± 5.5	5.5 ± 8.1	91
mouse fibroblast L cells				
control	88.0 ± 2.3[d]	6.6 ± 1.0	81.4 ± 2.0	
Ni^{2+}	6.0 ± 1.4	7.6 ± 1.2	—[e]	100
rat hepatoma Fu5AH cells				
control	35.4 ± 1.4[d]	6.6 ± 1.0	28.8 ± 0.7	
Ni^{2+}	6.4 ± 0.3	8.2 ± 0.6	—[e]	100

[a] Cells were incubated with ^{125}I-labeled α_2-M* in the presence or absence of nickel chloride (10 mM) for 4 h at 4 °C as described in Materials and Methods and Figure 1, washed, extracted in 0.1 N NaOH, and counted. The specific binding was obtained by subtracting nonspecific binding from total binding. [b] Inhibition (%) of specific binding of α_2-M* due to the addition of nickel chloride (10 mM) during ligand binding experiments. [c] Mean ± SD, $n = 6$. [d] Mean ± SD, $n = 3$. [e] Specific binding was too low to detect.

Table 4: Effect of Nickel on the Binding of Different Ligands to the LRP/α_2-MR

ligands	specific binding (ng bound/mg of protein)[a]		% inhibition
	control	nickel[b]	
methylamine-activated α_2-macroglobulin	42 ± 9	—[c]	100
trypsin-activated α_2-macroglobulin	22 ± 2	2 ± 2	93
Pseudomonas exotoxin A	4 ± 3	10 ± 2	none
low density lipoprotein	23, 27	61, 57	none

[a] The binding of different radiolabeled ligands to the SMC was performed in triplicate as described in Materials and Methods and Figure 1. Mean ± SD are reported. The binding studies of low density lipoproteins were performed in duplicate. [b] Ni^{2+} (10 mM) was included in the binding medium to study its effect on the specific binding of α_2-M*. Ni^{2+} did not increase the nonspecific binding of these ligands to cells. [c] Nonspecific binding was higher than specific binding.

the binding of RAP; both are known ligands of the LRP/α_2-MR.

Effect of RAP on α_2-M Binding to SMC.* To rule out significant binding of α_2-M* to the newly reported signaling receptor that is not inhibited by RAP (Misra et al., 1994a), we performed competition experiments on SMC. The specific binding of radiolabeled α_2-M* was decreased in the presence of increasing concentrations of unlabeled α_2-M*; 60% and 96% inhibition was observed at 1 and 27 nM α_2-M*, respectively. The specific binding of α_2-M* was also inhibited by unlabeled RAP. Fifty percent inhibition was observed at 13 nM, in agreement with other studies (Williams et al., 1992; Misra et al., 1994a). A 200- and 500-fold molar excess of RAP resulted in 90% and 96% inhibition, respectively, suggesting that majority of the binding of α_2-M* to SMC was to the LRP/α_2-MR.

Effect of Nickel on the Binding of α_2-M to the Purified LRP/α_2-MR.* Direct evidence for the interaction between nickel and LRP/α_2-MR was obtained by studying the binding of $^{63}Ni^{2+}$ to immobilized receptor (Figure 4). The amount in counts per minute (cpm) bound to LRP/α_2-MR was significantly higher than that bound to α_2-M* or pig serum albumin (compare panel 4A with 4B and 4C). The amount of $^{63}Ni^{2+}$ bound to α_2-M* was similar to that bound to pig serum albumin (compare panel 4B with 4C). To obtain picomoles of nickel bound per mole of LRP/α_2-MR, we first measured the amounts of different proteins bound to microtiter plates. The mean percent of LRP/α_2-MR, α_2-M*, and pig serum albumin bound to plates was 63, 78, and 74%, respectively, when applied at concentration of 0.12 μg/well in triplicate. Since the amount of nickel bound to pig serum albumin was significant, we also determined the moles of albumin bound in LRP/α_2-MR-, α_2-M*-, and pig serum albumin−coated wells using ^{125}I-labeled compounds. The amount of pig serum albumin bound when added at a concentration of 10 mg/mL was 242, 197, and 259 pmol in LRP/α_2-MR−, α_2-M*−, and pig serum albumin−coated wells, respectively. Thus the amount of nickel bound to pig serum albumin was subtracted from total counts bound to LRP/α_2-MR and α_2-M* and were plotted in panels 4D and 4F. The binding of nickel to LRP/α_2-MR exhibited a rectangular hyperbola (Figure 4D). The curve was analyzed using an equation ($y = a^*x/(b + x)$; a: B_{max}, b: K_d). Nickel bound to LRP/α_2-MR with a dissociation constant of 0.04 μM. The B_{max} value was 2.6. Scatchard analysis of the curve suggests that two to three moles of nickel bound to each mole of the receptor (Figure 4E). Next, a similar analysis was performed for the binding of nickel to α_2-M* (Figure 4F). The specific binding of nickel to α_2-M* was close to zero. The curve could not be analyzed by using the binding isotherm described above. These data suggest that purified LRP/α_2-MR has two to three binding sites for nickel and that α_2-M* does not interact with nickel.

To show that the interaction of Ni^{2+} with the LRP/α_2-MR results in the inhibition of the binding of α_2-M*, we studied the effect of Ni^{2+} on the purified receptor. The purified receptor was immobilized to microtiter plates and nonspecific binding sites present on the plate were blocked with dog serum albumin (which does not contain the high-affinity binding site for Ni^{2+} (Predki et al., 1992). First, binding of radiolabeled α_2-M* to the immobilized receptor was studied in the presence of different concentrations of Ni^{2+} (Figure 5). Ni^{2+} at 0.5 mM inhibited greater than 50% of the α_2-M* binding. Complete inhibition of specific binding was achieved at higher concentrations. Next, we studied the effect of nickel on RAP binding. Under these conditions, nonspecific binding of RAP was increased, as observed in Figure 2B, making it difficult to interpret the effect of Ni^{2+} on RAP binding to the receptor (data not shown). Then we studied the effect of nickel using the

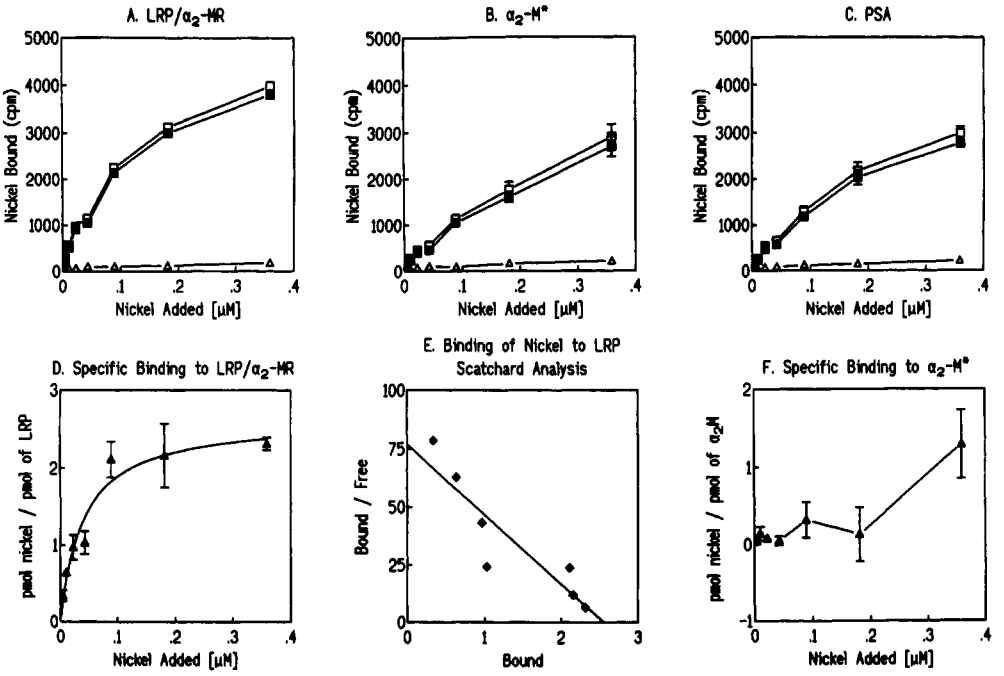

FIGURE 4: Binding of $^{63}Ni^{2+}$ to the purified LRP/α_2-MR. Microtiter wells were first coated in triplicate with 120 ng of purified receptor, α_2-M*, or pig serum albumin (psa) and then blocked with 1 mg of pig serum albumin per well as described in Materials and Methods. After washing, wells were incubated with different amounts of $^{63}NiCl_2$ for 18 h at 4 °C, washed, and counted. The cpm bound to LRP/α_2-MR, α_2-M*, and pig serum albumin are shown in panels A, B, and C, respectively. The open squares, closed squares, and open triangles represent total, specific, and nonspecific binding, respectively. Nonspecific binding was obtained using 50-fold excess of unlabeled nickel chloride. At most points, error bars are invisible because the standard deviations are smaller than symbols. Panel D is a nonlinear regression curve obtained for the binding of nickel to LRP/α_2-MR after subtracting the amount of nickel bound to pig serum albumin. Panel E is a linear (Scatchard) transformation of the curve generated in panel D. Panel F represents the specific binding of nickel to α_2-M* after subtracting the amount of nickel bound to pig serum albumin.

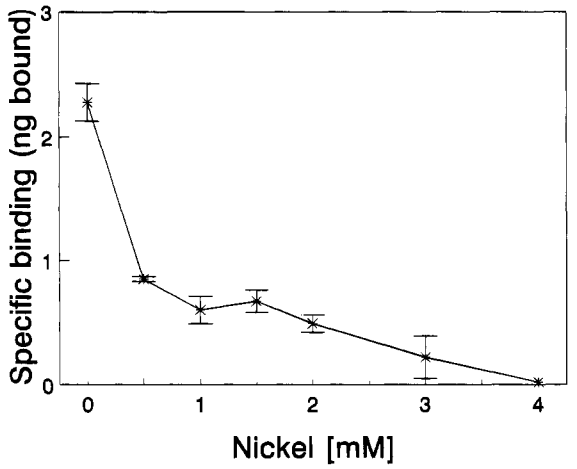

FIGURE 5: Effect of nickel on the binding of α_2-M* to the purified receptor. The purified receptor was immobilized on microtiter plates, and nonspecific binding sites were blocked by dog serum albumin as described in Materials and Methods. Triplicate microtiter wells were incubated overnight with ^{125}I-α_2-M* (1 nM) at 4 °C in the presence of different concentrations of nickel as indicated. For the determination of nonspecific binding 200-fold excess of unlabeled α_2-M* was used. Wells were washed and counted as described in Materials and Methods.

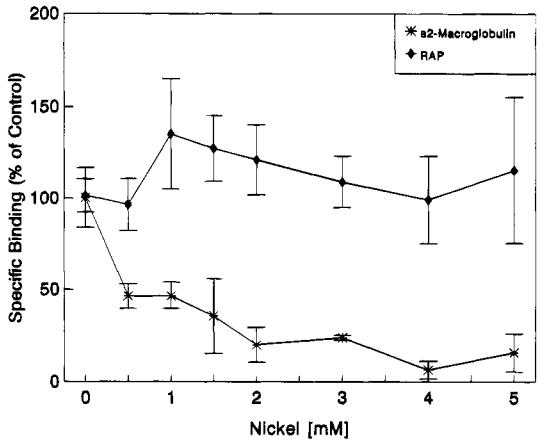

FIGURE 6: Effect of preincubation of the purified LRP/α_2-MR with nickel on the specific binding of α_2-M* and RAP. The purified receptor was immobilized on microtiter plates, and nonspecific binding sites were blocked by dog serum albumin as described in Materials and Methods. Microtiter wells were then incubated with different concentrations of Ni^{2+} for 8 h at 4 °C, washed once, and then incubated overnight with ^{125}I-α_2-M* (1 nM), ^{125}I-α_2-M* (1 nM) + unlabeled α_2-M* (0.2 μM), ^{125}I-RAP (13 nM), and ^{125}I-RAP (13 nM) + unlabeled RAP (1.3 μM) in triplicate. The wells were washed twice and eluted in 0.1 N NaOH and counted. Wells that were not treated with Ni^{2+} were used as controls representing 100% activity. Inhibition of specific binding by Ni^{2+} to the receptor is presented as a line (mean of triplicate determinations). The error bars represent standard deviation.

preincubation protocol described before SMC (Figure 3). Immobilized LRP/α_2-MR was incubated with different concentrations of Ni^{2+}, washed once to remove unbound Ni^{2+}, and then studied for the binding of α_2-M* and RAP (Figure 6). Increasing concentrations of Ni^{2+} inhibited the specific binding of α_2-M* to the purified LRP/α_2-MR immobilized on microtiter plates. In contrast, preincubation of the LRP/α_2-MR with Ni^{2+} followed by one washing did not inhibit the specific binding of RAP (Figure 6). Actually, a 10−20% increase in specific binding was observed. No increase in the nonspecific binding of α_2-M* and RAP was observed. As a control, wells were coated with dog serum albumin and the binding of radiolabeled α_2-M* and RAP was studied as described in Figure 6. The binding of α_2-

M* and RAP to dog serum albumin was 3- and 10-fold lower, respectively, when compared to their binding to the purified LRP/α_2-MR. The binding of these ligands to dog serum albumin was not inhibited by preincubation with Ni^{2+}. Next, we studied the effect of nickel on the binding of *Pseudomonas* exotoxin A to the purified receptor. The binding of radiolabeled exotoxin A to the immobilized purified receptor was inhibited >80% by RAP (20 μg/mL) but was not inhibited by the presence of 5 mM nickel. These results suggest that Ni^{2+} is a site-specific inhibitor for the binding of the α_2-M* binding sites present on the LRP/α_2-MR.

DISCUSSION

The LRP/α_2-MR is a multifunctional receptor that recognizes several ligands. Here, we show for the first time that Ni^{2+} specifically inhibits the binding of α_2-M* to the LRP/α_2-MR (see tables and Figures 1 and 5). The inhibition could be due to the interaction of nickel with α_2-M* or LRP/α_2-MR. Two lines of evidence suggest that the effect of nickel was not due to its interaction with α_2-M*: Preincubation of α_2-M* with nickel had no effect on the binding to LRP/α_2-MR (data not shown), and $^{63}Ni^{+2}$ did not bind to immobilized α_2-M* (Figure 4). Our observation that nickel does not interact with α_2-M* is in agreement with Pratt and Pizzo 1984), who studied the effects of divalent cations on the structure and function of α_2-macroglobulin. They showed that zinc, copper, and mercury decrease the proteinase inhibitory activity of α_2-macroglobulin by dissociating the active tetramer complex into monomeric subunits. In contrast, barium, cadmium, and nickel had no effect on the structure and function of native and activated α_2-macroglobulin (Pratt & Pizzo, 1984). Evidence that nickel interacts with LRP/α_2-MR was obtained by studying the binding of $^{63}Ni^{+2}$ to immobilized LRP/α_2-MR (Figure 4). These experiments are not conclusive because of the high nonspecific binding of nickel to pig serum albumin, the estimation of the amount of protein bound to microtiter plates, and the assumption concerning the correct orientation of the bound molecules. However, they did indicate that the binding of nickel to LRP/α_2-MR was significantly higher than pig serum albumin binding, and under similar conditions no binding of nickel to α_2-M* was observed. Furthermore, preincubation of LRP/α_2-MR with nickel resulted in the inhibition of subsequent binding of α_2-M* (Figure 6), suggesting that interactions between nickel and LRP/α_2-MR are the determining factors in the inhibition of α_2-M* binding.

In contrast to its effect on α_2-M* binding, nickel does not inhibit RAP and *Pseudomonas* exotoxin A binding to the LRP/α_2-MR. The lack of nickel effect on RAP binding could be due to high-affinity binding of RAP at several sites on the LRP/α_2-MR (Williams et al., 1992; Iadonato et al., 1993). Particular attention was given to partial inhibition of RAP binding by nickel, but none was observed (Figure 6). In fact, increased binding of RAP to the receptor was consistently observed (Figures 2 and 6). These studies suggest that binding of RAP to purified LRP/α_2-MR is not affected by nickel. Similarly, no inhibition for the binding of exotoxin A to the receptor was observed. Hence, nickel appears to be a specific inhibitor only for the α_2-M* binding site.

The effect of Ni^{2+} is probably not due to displacement of all calcium ions from the receptor. First, calcium has a higher affinity for the receptor than does Ni^{2+} (Moestrup et al., 1990). The effect of Ni^{2+} was observed in the presence of high concentrations of calcium (5mM); if Ca^{2+} and Ni^{2+} competed for the same sites, Ni^{2+} could not have exerted its effect. Second, Ni^{2+} affects only the binding of α_2-M*; in contrast, Ca^{2+} is required for the binding of all ligands (Brown et al., 1991; Herz, 1993; Krieger & Herz, 1994; Moestrup, 1994; Strickland et al., 1995). Third, preincubation of SMC or purified receptor with Ni^{2+} in the presence of 5 mM $CaCl_2$ followed by incubation with calcium without Ni^{2+} resulted in the inhibition of α_2-M* binding (Figures 3 and 6), suggesting that Ni^{2+} probably binds to a site different from a Ca^{2+} binding site. Our experiments, however, do not rule out the possibility that one or more Ca^{2+} ions present at the α_2-M* binding site on the receptor might have been displaced by Ni^{2+}.

The specific inhibition of α_2-M* binding by Ni^{2+} suggests that the binding domain for the α_2-M* is probably separate from other ligand binding domains. For instance, the binding sites for the α_2-M* and RAP and exotoxin A are sufficiently separate and different from each other that Ni^{2+} can differentiate between these sites. Ni^{2+} may inhibit the binding of α_2-M* by direct competition for the same site or by inducing conformational changes within the α_2-M* binding domain. The specificity of nickel action may be helpful in defining the binding site for α_2-M* and in addressing ligand−receptor interactions.

The primary sequences of Ni^{2+} binding domains in different proteins are rather heterogeneous. In albumin, the three amino-terminal residues, Asp−Ala−His, and the free NH_2-group, form the binding site for copper and Ni^{2+} (Predki et al., 1992; Peters & Blumenstock, 1994). In the LRP/α_2-MR, however, the amino-terminal end is blocked, and the amino-terminal sequence is Ala-Ile-Asp (Herz et al., 1988), suggesting that the nickel-binding site in the LRP/α_2-MR is different from that of albumin. Histidine-rich nickel-binding clusters in the estrogen-regulated serpin of *Xenopus* (Sunderman, 1993), the diphtheria toxin (Wang et al., 1994), and the nickel-binding protein of *Rhizobium leguminosarum* (Rey et al., 1994) are absent in the LRP/α_2-MR. Similarly, cysteine-rich nickel-binding domains observed in nickel hydrogenases (Albracht, 1993; Rey et al., 1994), which oxidize H_2 generated by different enzymes such as nitrogenase complex during N_2-fixation, are not present in the LRP/α_2-MR. Thus, the amino acid sequence of the nickel-binding site in the LRP/α_2-MR may be new or could be a variant of other heterogeneous sequences.

In summary, we have provided evidence that the binding of Ni^{2+} to the LRP/α_2-MR results in the inhibition of α_2-M* binding to the receptor but has no effect on RAP and exotoxin A binding. These studies suggest that nickel may be a specific inhibitor for the α_2-M* binding site as opposed to the RAP, which is a general inhibitor for the receptor. The physiologic significance of the inhibition of α_2-M* binding remains to be determined. However, the small size, low cost, easy availability, and high specificity of its action suggest that Ni^{2+} may be a valuable and an inexpensive tool to investigate ligand binding domains present on the LRP/α_2-MR.

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