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Articles

Nickel Inhibits Binding of α_2-Macroglobulin-Methylamine to the Low-Density Lipoprotein Receptor-Related Protein/α_2-Macroglobulin Receptor but Not the α_2-Macroglobulin Signaling Receptor†

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ABSTRACT: A previous study demonstrated that activated α_2-macroglobulin (α_2M*) binding to the low-density receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR) is blocked by Ni^{2+} [Hussain, M. M., et al. (1995) *Biochemistry 34*, 16074−16081]. We now report that the effect of Ni^{2+} is on a region of the α_2M molecule upstream of the carboxyl terminal receptor recognition domain. This observation is consistent with previous observations from this laboratory suggesting that α_2M* binding to LRP/α_2MR involves a region of the α_2M molecule immediately upstream of the receptor recognition domain [Enghild, J. J., et al. (1989) *Biochemistry 28*, 1406−1412]. We further demonstrate that Ni^{2+} has no effect on the binding of α_2M* or a cloned and expressed receptor binding fragment (RBF) to the recently described α_2M signaling receptor as assessed by direct binding and signal transduction studies.

The α-macroglobulins are part of a large super family including complement components C_3 and C_4 as well as human α_2-macroglobulin (α_2M)[1] and so called pregnancy zone protein [for reviews, see Sottrup-Jensen (1987) and Chu and Pizzo (1994)]. These proteins generally contain an internal β-cysteinyl-γ-glutamyl thiolester as well as a proteinase sensitive region. Proteolytic cleavage of human α_2M or direct nucleophilic attack on the thiolesters triggers a major conformational change which exposes receptor recognition sites present in each of the four α_2M subunits [for reviews, see Salvesen and Pizzo (1993) and Chu and Pizzo (1994)].

Two receptors bind α_2M*, namely, LRP/α_2MR and α_2-MSR. LRP/α_2MR is a scavenger receptor which binds a wide variety of ligands including α_2M*, RBF, lactoferrin, lipoprotein lipase, and *Pseudomonas* exotoxin A (Strickland et al., 1990; Kristensen et al., 1990) [for review, see Krieger and Herz (1994)]. RAP blocks binding of all known ligands to LRP/α_2MR; however, most of the other ligands which bind to this receptor appear to interact with independent domains and do not show cross-competition (Strickland et al., 1990; Krieger & Herz, 1994). Binding of α_2M* to LRP/α_2MR is followed by uptake and degradation in lysozomes, but not activation of a signaling cascade.

By contrast, binding of α_2M* or RBF to α_2MSR triggers classical signaling cascades as well as regulating cell proliferation (Misra et al., 1993; 1994a,b; 1995; 1997; Howard et al., 1996a,b; Webb et al., 1995). While RAP blocks binding of α_2M* and RBF to LRP/α_2MR, it has no effect on binding of these ligands to α_2MSR. The affinity of these ligands is also quite different being of extremely

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_2M*, activated α_2M; HHBSS, Hanks' balanced salt solution; LDL, low-density lipoprotein, LRP/α_2MR, the low-density lipoprotein receptor-related protein/α_2M receptor; RAP, receptor-associated protein; RBF, receptor binding fragment; Fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; IP_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, the concentration of intracellular Ca^{2+}; *cis*-DDP, *cis*-dichlorodiammine-platinum(II).

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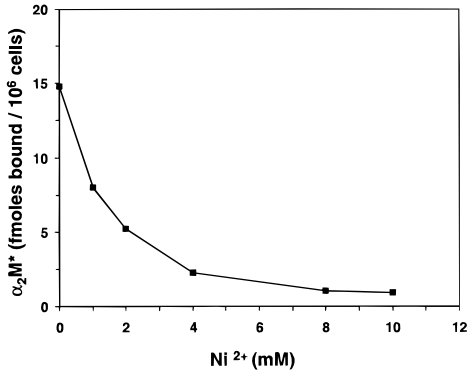

FIGURE 1: The effect of increasing concentrations of Ni^{2+} on the binding of α_2M* to macrophages. The specific binding of [^{125}I]α_2M* (0.5 nM) in the presence of increasing concentrations of Ni^{2+} was studied employing murine peritoneal macrophages which express both α_2M receptors, LRP/α_2MR and α_2MSR (Misra et al., 1994a,b; Howard et al., 1996a−c).

high affinity to the signaling receptor ($K_d \approx 50$ pM) and of moderately high affinity to the scavenger receptor ($K_d \approx 5$ nM).

Recently, Hussain et al. (1995) have reported that Ni^{2+} blocks binding of α_2M* to LRP/α_2MR. These studies did not examine the effects of Ni^{2+} on the binding of α_2M* to the signaling receptor. Moreover, these investigators did not study RBF binding to either receptor. In the present report, we demonstrate that Ni^{2+} blocks binding of α_2M*, but not RBF, to LRP/α_2MR. Ni^{2+} did not affect binding of either ligand to α_2MSR and had no effect on the signaling cascade activated after α_2M* or RBF binds to this receptor.

MATERIALS AND METHODS

Materials. α_2M was purified, characterized, activated with methylamine and radiolabeled with ^{125}I as previously described (Imber & Pizzo, 1981). This receptor-recognized form of α_2M is referred to as α_2M* in the remainder of the text. The receptor binding fragment was cloned and expressed in *Escherichia coli*, characterized, and radiolabeled with ^{125}I as previously reported (Salvesen et al., 1992; Howard et al., 1996b,c). Fura-2/AM was purchased from Molecular Probes, Inc. (St. Louis, MO). 2-[^{3}H]*myo*-inositol (specific activity, 10−20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). RAP was prepared as previously described (Howard et al., 1996a). The pGEX 39 kDa expression construct was a kind gift of Dr. Joachim Herz (University of Texas, Southwestern, Dallas, TX). All other reagents were of the highest grade available.

Ligand Binding Assays. Thioglycolate-elicited macrophages were obtained from C57 BI/6 mice (Charles Rivers Laboratories, Raleigh, NC) by peritoneal lavage as previously described (Misra et al., 1993, 1994a,b). The cells were plated in 48-well plates to achieve a density of 1×10^6 cells/well. The monolayers were washed three times in ice-cold Hanks' balanced salt solution containing 25 mM HEPES (HHBSS) and 12.5 units/mL penicillin, 6.5 μg/mL strepomycin, and 5% bovine serum albumin. Nonspecific binding was assessed by incubating some of the wells in HHBSS without Ca^{2+} or Mg^{2+} containing a 100-fold excess of either unlabeled α_2M* or RBF. Increasing concentrations of ^{125}I-ligands (α_2M* or RBF) were added to the wells which were then incubated for 16−18 h at 4 °C as previously described (Howard et al., 1996a,b). When the effect of Ni^{2+} was

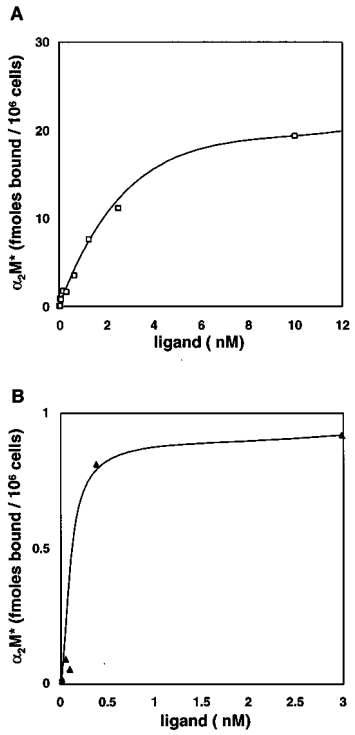

FIGURE 2: The effect of Ni^{2+} on the binding of α_2M* to macrophage LRP/α_2MR and α_2MSR. [^{125}I]α_2M* specific binding as a function of ligand concentration was examined in the presence of Ni^{2+} at a fixed concentration, 5 mM. Panel A shows the binding isotherm in the absence of Ni^{2+} and panel B in the presence of Ni^{2+}. The *y*-axis in panel B has been greatly expanded in comparison to panel A so that the specific binding in the presence of Ni^{2+} can be discerned. Details of the binding assay are contained in the Materials and Methods.

studied, the metal ion was present during the entire incubation period. Concentrations of Ni^{2+} between 1 and 10 mM were studied. All binding studies were performed at least three to five times and in triplicate. Standard errors of the mean for these studies were ≤10%. Scatchard analysis was used to analyze the binding data as previously reported (Howard et al., 1996a,b).

In one series of studies, the specific binding of α_2M* (0.5 nM) was studied at increasing concentrations of Ni^{2+}. These studies were performed at 4 °C for cells which were incubated with or without Ni^{2+} for 16−18 h. The study was performed on five occasions in triplicate. Standard errors of the mean for these studies were ≤10%.

IP_3 and Ca^{2+} Measurements. IP_3 was quantified as described by Berridge (1983) and previously reported in detail (Misra et al., 1993, 1994a,b; Howard et al., 1996a,b). In brief, macrophage monolayers were incubated with 2-[^{3}H]-*myo*-inositol (8 μCi/mL) in each well at 37 °C for 16−18 h. Monolayers were then rinsed three times in HHBSS containing 1 M$CaCl_2$, 1 mM $MgCl_2$, 10 mM LiCl, pH 7.4. The cells were then incubated in this medium with ligand for various time periods. The reaction was stopped by removing the medium and adding 6.25% perchloric acid solution. The cells were placed in tubes containing 1 mL of octylamine/Freon (1:1 v/v) containing 5 mM EDTA. The tubes were then centrifuged at 5600*g* for 20 min at 4 °C. The upper phase was applied to a 1 mL column of AG1-X8 formate Dowex resin (Bio-Rad Laboratories, Richmond, CA). The column was eluted serially with batches of ammonium formate solution containing 0.1 M formic acid as described

(Misra et al., 1993). Aliquots were then counted by liquid scintillation. For studies of the effect of Ni^{2+}, the metal ion was present at 5 mM for the time period of incubation of the cells with α_2M*.

For measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), marophages were plated on glass coverslips placed in 35 mm Petri dishes at a density of 1.5×10^5 cells/cm^2 and incubated in RPMI medium for 16−18 h in a humidified incubator at 37 °C with 5% CO_2. The cells were then treated with Fura-2/AM (1−1.5 μM) containing medium at room temperature for 30 min in the dark. The monolayers were then washed twice in HHBSS containing 75 μM Ca^{2+}. Under these conditions, macrophage capacitative entry of Ca^{2+} is minimal when the cells are exposed to α_2M* (Misra et al., 1993). These conditions were employed to allow the effect of Ni^{2+} to be studied. Ni^{2+} is capable of blocking uptake of Ca^{2+} from the extracellular medium; however, this cation does not penetrate the cells and does not affect the mobilization of Ca^{2+} from intracellular pools (Kwan & Putney, 1990). For studies of the effect of Ni^{2+}, the cation was present at a concentration of 5 mM during the incubation of cells with α_2M*.

Measurements of $[Ca^{2+}]_i$ were obtained employing a digital imaging microscope as described in detail elsewhere (Misra et al., 1993). A baseline measurement in buffer was obtained for the macrophage monolayers at 37 °C before adding α_2M*. When Ni^{2+} (5 mM) was employed in studies, it was present in the buffer prior to addition of the α_2M*. $[Ca^{2+}]_i$ was then monitored at 37 °C. Ni^{2+} at this concentration had no effect on trypan blue exclusion compared to control cells similarly treated for periods of at least 15 min at 37 °C, a time sufficient for completion of IP_3 and $[Ca^{2+}]_i$ measurements. This observation is in accord with previous studies (Kwan & Putney, 1990).

RESULTS

*Effect of Ni^{2+} on the Cellular Binding of α_2M**. Consistent with previous observations employing purified LRP/α_2MR or smooth muscle cells (Hussain et al., 1995), Ni^{2+} blocks the binding of [^{125}I]α_2M* (0.5 nM) to murine peritoneal macrophages (Figure 1). In the present studies, the maximal effect of Ni^{2+} was achieved at a $[Ni^{2+}]$ of about 5 mM. This concentration was employed in the subsequent binding studies. The specific binding of [^{125}I]α_2M* never approached zero in any of our studies.

The binding of [^{125}I]α_2M* as a function of concentration was then studied in the absence or presence of Ni^{2+} (Figure 2). The binding of [^{125}I]α_2M* was suppressed to extremely low levels at all concentrations of [^{125}I]α_2M* studied in the presence of Ni^{2+} (5 mM) (Figure 2). In order to analyze the data in greater detail, Scatchard analysis was then employed. As can be seen, the effect of Ni^{2+} was restricted to the lower affinity site ($K_d \approx 10$ nM) (Figure 3) previously identified as LRP/α_2MR (Howard et al., 1996a,b,d). Binding to the very high affinity α_2M* binding site ($K_d \approx 50$ pM) was unaffected. This class of binding sites has previously been identified as α_2MSR (Howard et al., 1996a−c). This effect is best seen by comparing the effect of two Ni^{2+} concentrations, 1 and 5 mM on the two classes of binding sites (Figure 3, panels B and C). Only the lower affinity class of sites shows dose-dependent suppression of the binding of [^{125}I]α_2M* (Figure 3, panels B and C).

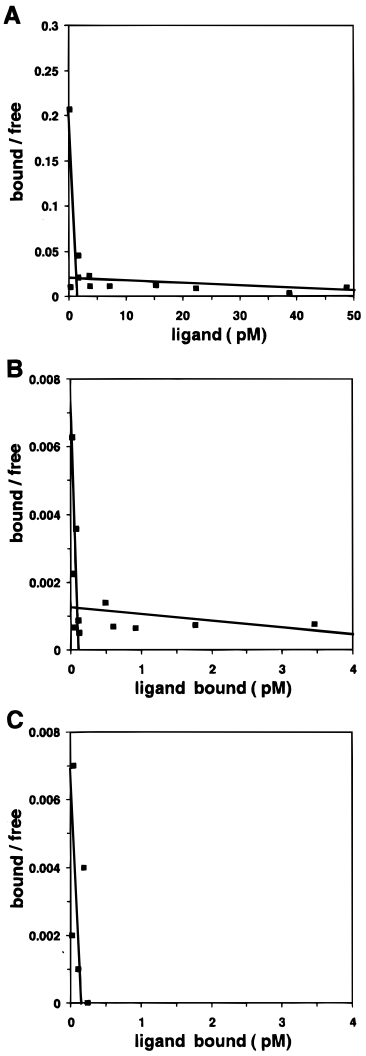

FIGURE 3: Scatchard analysis of the binding of α_2M* in the presence of Ni^{2+} to macrophage LRP/α_2MR and α_2MSR. The Scatchard replots are for the data in Figure 2 as well as for a study in the presence of 1 mM Ni^{2+}. Panel A represents the data obtained in the absence of Ni^{2+}, panels B and C in the presence of a $[Ni^{2+}]$ of 1 and 5 mM, respectively. The *y*-axis in panels B and C have been significantly expanded to better discern the effect of Ni^{2+} on the binding of [^{125}I]α_2M* to LRP/α_2MR (lower affinity site). Details of the binding assay are contained in the Materials and Methods.

Effect of Ni^{2+} on the Cellular Binding of RBF. The effect of Ni^{2+} on the binding of RBF to LRP/α_2MR and α_2MSR was next investigated (Figure 4). In contrast to the observations made when α_2M* was the ligand, Ni^{2+} had no effect on the binding of [^{125}I]RBF to LRP/α_2MR. [^{125}I]RBF binding to α_2MSR was also unaffected by Ni^{2+}. The observations made with respect to the binding of RBF to LRP/α_2MR suggests that a portion of the LRP/α_2MR receptor binding site is present in a region of α_2M upstream of RBF. This observation is consistent with a significant number of previous observations from this laboratory suggesting a similar conclusion (Pizzo et al., 1986; Enghild et al., 1989; Isaacs et al., 1988; Roche et al., 1988; Howard et al., 1996a).

Effect of Ni^{2+} on IP_3 Synthesis and $[Ca^{2+}]_i$. The effect of Ni^{2+} on the ability of α_2M* to induce IP_3 synthesis by ligation of macrophage α_2MSR was then studied (Figure 5). At a $[Ni^{2+}]$ of 5 mM there was no decrease in IP_3 synthesis induced by α_2M* (100 pM). The shape of the curve was, however, somewhat different with a higher peak value and prolonged increase in IP_3 synthesis above the basal level.

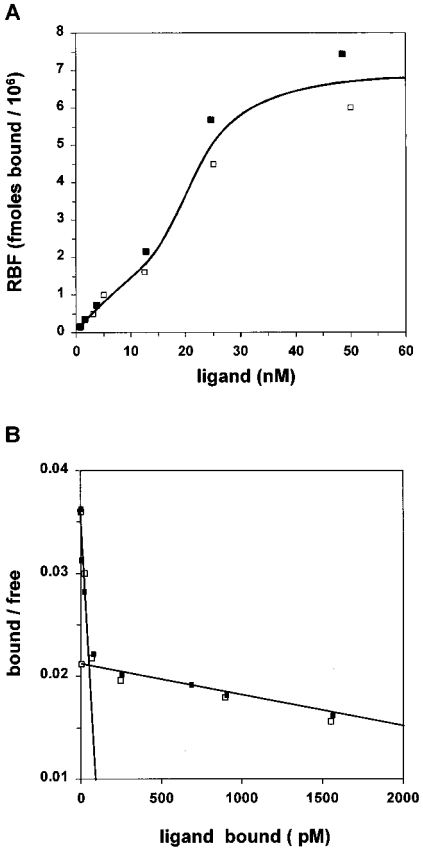

FIGURE 4: The effect of Ni^{2+} on binding of RBF to macrophage LRP/α_2MR and α_2MSR. Panel A shows the binding of [^{125}I]RBF macrophages in the presence of Ni^{2+} at a fixed concentration of 5 mM (■). The binding curve was essentially identical to that obtained in the absence of Ni^{2+} (□). Panel B shows the Scatchard replot of the data from panel A. Details of the binding assay are contained in the Materials and Methods.

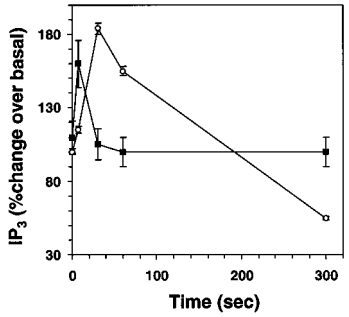

FIGURE 5: The effect of Ni^{2+} on IP_3 synthesis induced in macrophages by α_2M*. Macrophages were exposed to α_2M* (100 pM) in the absence (■) or presence of (○) a fixed concentration of Ni^{2+} (5 mM). An IP_3 dose-response curve with α_2M* in the range 0−60 pM was also obtained in the presence and absence of Ni^{2+} at a fixed concentration of 5 mM. These curves were identical and are not shown. The values reported are from three separate experiments performed in triplicate. The values are shown as the mean ± the standard error of the mean.

Ligation of α_2MSR by α_2M* or RBF causes a 2−3-fold IP_3-dependent rise in macrophage $[Ca^{2+}]_i$ (Misra et al., 1993, 1994a,b). The ability of α_2M* (100 pM) to induce an increase in macrophage $[Ca^{2+}]_i$ was, therefore, examined in the presence or absence of Ni^{2+} (5 mM). The absolute rise in $[Ca^{2+}]_i$ was comparable and greater than 2-fold in each case (Table 1). While the absolute increase in $[Ca^{2+}]_i$ was comparable in the presence and absence of Ni^{2+}, we also observed a more prolonged response to α_2M* in the presence of Ni^{2+}. This is difficult to quantify or represent since it is based on the visualization of many individual cells. However, visualizing at least 100 cells for each experimental group suggests that the duration of the increase in $[Ca^{2+}]_i$ induced by α_2M* in the presence of Ni^{2+} is 2−3-fold greater in duration than the increase induced by α_2M* alone.

Table 1: Effect of Ni^{2+} on the Ability of α_2M* to Stimulate Increases in $[Ca^{2+}]_i$ in Macrophages

treatment[a]	basal	ligand-induced	fold increase
α_2M*	153.7 ± 11.3	347.9 ± 22.1	2.26
α_2M* + Ni^{2+}	117.0 ± 4.9	262.8 ± 33.6	2.25

[a] Macrophages were treated with α_2M* (100 pM) in the absence or presence of Ni^{2+} (5 mM). The peak increase in $[Ca^{2+}]_i$ is indicated for cells exposed to ligand and the baseline before ligand addition is indicated as the basal level. The stimulated values represent the peak response seen between 1 and 2 min after the cells were exposed to α_2M* with or without Ni^{2+}. At least 100 cells were studied on several different days for both experimental conditions. The values reported are the mean ± the standard error of the mean. The results are comparable to previous data reported from our laboratory (Misra et al., 1993; 1994a,b).

The effect of Ni^{2+} (5 mM) on the ability of RBF to induce both macrophage IP_3 synthesis and increases in $[Ca^{2+}]_i$ was also studied. As with α_2M*, Ni^{2+} had no effect on either IP_3 synthesis or $[Ca^{2+}]_i$ induced by RBF (data not shown).

DISCUSSION

Two distinct receptors bind receptor-recognized forms of α_2M; namely, LRP/α_2MR and α_2MSR. Binding of α_2M* to LRP/α_2MR is clearly an important catabolic pathway for uptake of α_2M−proteinase complexes (Salvesen & Pizzo, 1993; Kriger & Herz, 1994; Chu & Pizzo, 1994). It has been estimated that during a single day humans turn over as much as a gram of α_2M by virtue of proteinase activation and uptake of the complexes by the liver and other organs (Salvesen & Pizzo, 1993). The role of α_2MSR is less clearly understood since this receptor was only recently identified (Misra et al., 1994a,b). Ligation of this receptor regulates signal transduction cascades in a variety of cells. These events are linked to DNA synthesis and cellular proliferation of both smooth muscle and rheumatoid synovial cells (Webb et al., 1995; Misra et al., 1997). These effects are seen at extremely low concentrations of ligand, reflecting the very high affinity of α_2M* or RBF for α_2MSR ($K_d \approx 50$ pM).

In the present study, we demonstrate that Ni^{2+} blocks the binding of α_2M* to LRP/α_2MR but not α_2MSR. Previous investigators have demonstrated that Ni^{2+} blocks the binding of α_2M* to LRP/α_2MR (Hussain et al., 1996); however, these investigators did not examine the effect of Ni^{2+} on α_2MSR or signal transduction. While Ni^{2+} did not affect the binding of α_2M* to α_2MSR, we did find some effect of Ni^{2+} on the ability of α_2M* to promote IP_3 synthesis and increases in $[Ca^{2+}]_i$. This effect took the form of a prolonged increase in both IP_3 synthesis and the rise in $[Ca^{2+}]_i$ induced by α_2M* in the presence of Ni^{2+}. The reason for this effect is unclear. In part, it may reflect the fact that, by blocking LRP/α_2MR-mediated uptake of α_2M*, receptor occupancy of α_2MSR may be prolonged. By analogy, it has been shown that LRP/α_2MR, by virtue of its ability to bind urinary-type plasminogen activator (u-PA), also affects receptor occupancy of the u-PA receptor, UPAR. Ligation of UPAR promotes DNA synthesis and cellular proliferation by activation of a signaling cascade (del Rosso et al., 1993; Dumler et al.,

1993). This receptor is not capable of promoting ligand uptake which appears to require the coexpression of LRP/α_2MR on cells (Grobmyer et al., 1993). LRP/α_2MR is capable of binding and promoting uptake of the u-PA/UPAR complex thus terminating a signaling event. This appears to be the primary mechanism for regulating the growth factor activity of u-PA.

In contrast to the effect of Ni^{2+} on α_2M*, Ni^{2+} had no effect on the binding of RBF to LRP/α_2MR. The effect, therefore, of Ni^{2+} on α_2M* may involve a region of the protein upstream from the carboxy terminal receptor recognition domain. This observation is of interest in view of previous studies from this laboratory with regard to the effects of *cis*-dichlorodiamineplatinum(II) (*cis*-DDP) on the receptor recognition of α_2M*. *cis*-DDP affects amino acid residues which are not in the carboxyl terminal receptor recognition domain (Roche et al., 1988; Enghild et al., 1989; Howard et al., 1996a), but in a region which is upstream of the receptor binding domain (Enghild et al., 1989). While RBF does incorporate a small amount of *cis*-DDP (Enghild et al., 1989), this reaction also has no effect on the binding of RBF to α_2MSR (Howard et al., 1996a). Similarly oxidation of RBF does not alter its interaction with α_2MSR (Wu & Pizzo, 1997). It is of interest that exposure of RBF to Ni^{2+} also has no effect on its interaction with α_2MSR.

The effect of Ni^{2+} on the binding of ligands to LRP/α_2-MR appears restricted to α_2M* since Hussain et al. (1995) found no effect of Ni^{2+} on the binding of *Pseudomonas* exotoxin A, LDL, or RAP to this receptor. We have confirmed these observations and also have extended them to lipoprotein lipase and lactoferrin with similar results (U. K. Misra, and S. V. Pizzo, unpublished observations). Hussain et al. (1995) demonstrated that LRP/α_2MR bound Ni^{2+} and they concluded that the Ni^{2+} effect was the result of binding this cation to the receptor. Our data obtained with RBF suggest that this hypothesis may need to be reconsidered. Clearly, RBF and α_2M* bind to the same site on LRP/α_2MR (Enghild et al., 1989; Howard et al., 1996a−d). The lack of a Ni^{2+} effect on RBF binding to LRP/α_2-MR may suggest that binding of Ni^{2+} to α_2M*, rather than LRP/α_2MR, accounts for the loss of binding of α_2M* to LRP/α_2MR in the presence of Ni^{2+}.

The lack of an effect of Ni^{2+} on LDL binding to LRP/α_2MR may also be inconsistent with the original hypothesis. We have recently demonstrated that LDL and α_2M* bind to the same domain on LRP/α_2MR (Wu & Pizzo, 1996). This is unusual in that with the exception of RAP, few ligands cross-compete for binding to LRP/α_2MR (Krieger & Herz, 1994). Since LDL and α_2M* both bind to the same domain on LRP/α_2MR (Wu & Pizzo, 1996), the reason for the differential effect of Ni^{2+} on the binding of these two ligands to this receptor is unclear assuming that the Ni^{2+} effect is dependent on the binding of this metal ion to LRP/α_2MR. The study of Hussain et al. (1995) did show binding of Ni^{2+} to α_2M* as well as to LRP/α_2MR. Moreover, we have previously employed Ni^{2+} chelate chromatography to purify a number of α-macroglobulins including bovine, human, chicken, and frog (Feldman et al., 1984; Feldman & Pizzo, 1985, 1989a,b). Whether this binding accounts for the differential effect of Ni^{2+} on RBF and α_2M* binding to LRP/α_2MR will require further investigation.

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