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# Dimethyl Sulfoxide Decreases Specific EGF Binding

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Dimethyl sulfoxide (DMSO) stimulates tyrosine phosphorylation of the hepatic EGF receptor in isolated membrane preparations. To determine whether DMSO affects EGF binding, primary cultures of rat hepatocytes were incubated with 1–10% DMSO for 30 min prior to the addition of $^{125}$I-EGF. DMSO (1–2%) reduced specific $^{125}$I-EGF binding; the effect was maximal (a 40–60% reduction) at 5–7.5% DMSO and was reversed by removing the DMSO. Scatchard analysis showed that the reduction in binding was due to a change in receptor affinity. The decrease in binding was not seen when other, slightly less polar, solvents (eg, acetone and ethanol) were tested. DMSO also reduced $^{125}$I-EGF binding to purified rat liver plasma membranes. This reduction was seen in the absence of added ATP and in membranes that had been pretreated with TLCK, a tyrosine kinase inhibitor. Thus, completion of the receptor autophosphorylation reaction was not necessary to effect the change. The data are consistent with a DMSO-induced alteration of receptor conformation that reversibly reduces receptor affinity.

**Key words: receptor affinity, epidermal growth factor, membrane proteins, rat hepatocytes, solvents**

Dimethyl sulfoxide (DMSO), a highly polar solvent, has protean effects on cellular physiology and biochemistry [1,2]. These include alterations in the activity of enzymes [3–7], cell and cytoskeletal structure [8–10], lymphocyte immunoglobin capping [11], and ligand-receptor interaction [12]. Several complex time-dependent processes also are affected by DMSO, notably the differentiation of certain neoplastic cell lines. This was first observed in Friend murine erythroleukemia cells [2,13] and subsequently has been reported using human promyelocytic leukemia cells [14], neuroblastoma cells [15], macrophages [16], and hepatocytes [17]. The mechanism by which these diverse effects are brought about remain largely undefined; however, changes in membrane properties [18] and in the function of membrane proteins [19,20] have been implicated.

Abbreviations used are: DMSO, (dimethyl sulfoxide); EGF, (epidermal growth factor), TLCK (tosyl-l-lysine chloromethyl ketone).

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This laboratory investigated a new mechanism by which DMSO may regulate membrane function, ie, stimulation and inhibition of specific membrane protein kinase activities [7,21,22]. The most selective effect noted to date has been the stimulation of epidermal growth factor (EGF) receptor tyrosine phosphorylation. The phosphorylation of the EGF receptor by an EGF-dependent tyrosine-specific kinase was first reported by Carpenter, Cohen, and co-workers [23,24], and recent data indicate that the kinase responsible is intrinsic to the 170,000 dalton glycoprotein receptor [25]. Other hormone and growth factor receptors also exhibit a similar kinase activity [26–28].

Because DMSO also stimulated EGF receptor tyrosine phosphorylation in Triton X-100 solubilized preparations [21], it appears that the solvent can act directly on this receptor, changing it to a conformation favorable for tyrosine phosphorylation. This study tested whether the putative conformational change produced by DMSO in membranes and solubilized preparations would be reflected in an alteration in EGF binding to intact cells.

## MATERIALS AND METHODS

Type II collagenase was purchased from Worthington Biochemicals. Waymouth-M-752/1 medium was obtained from GIBCO. Purified insulin was a gift from the Eli Lilly Company, and $^{125}I$ Na was purchased from Amersham. Tosyl-l-lysine chloromethyl ketone (TLCK) and other reagents were obtained from Sigma Co.

### Preparation of Isolated Hepatocytes

Male Sprague-Dawley rats (250–300g) from Charles River were maintained in quarters lighted from 7:00 am to 7:00 pm and fed and watered ad libitum. Hepatocytes were prepared between 9:00–11:00 am [29]. The portal vein was cannulated and a perfusion was begun with oxygenated, sterile isotonic saline/$NaHCO_3$ pH 7.4 37°C (7 min), followed by 10 min (12–14 ml/min) perfusion with 60 units/ml collagenase in 4mM $CaCl_2$. The liver was excised, the capsule cut, and the cells dispersed in collagenase-containing solution. After straining through a sterile wire mesh and low-speed centrifugation, the cells were resuspended in isotonic NaCl/$NaHCO_3$ containing 1% albumin and purified by gravity sedimentation. The viability of the purified hepatocytes was 80–90%.

Insulin 100nM and 1% fetal bovine serum were added to the medium, and the hepatocytes were plated at 1–1.5 $\times$ $10^6$ cells per 35mm Falcon 3001 plates. Cells were maintained at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. After 3–4 hr, unattached cells were removed and the medium changed to Waymouth's containing 100 nM insulin alone.

A431 cells derived from human epidermoid carcinoma (originally obtained from Dr. G. Todaro) were grown to confluence in Limbro 24 well trays in 2 $cm^2$ wells in the presence of MEM and 10% fetal calf serum. The binding in both cell types was assessed as detailed below.

### Preparation of $^{125}$EGF

EGF was purified to homogeneity from the submaxillary glands of adult male COBS mice CD-1 (Charles River Breeding) using the method of Savage and Cohen [30]. The iodination of EGF was performed by the method of Hunter and Greenwood

[31] using chloramine T and was stopped after 60 sec with sodium metabisulfite. The mixture was resolved into $^{125}$I-EGF and unreacted $^{125}$I fractions by a Sephadex G-15 column eluted with 0.1 M sodium phosphate pH 7.4 containing 0.1% BSA. The $^{125}$I-EGF product was stored frozen in the eluting buffer at specific activity of 5–8 × $10^5$ cpm/ng EGF.

### $^{125}$I-EGF Binding

Total $^{125}$I-EGF binding for each sample was determined in triplicate; the nonspecific binding for each sample measured in the presence of 1 μg unlabeled purified EGF was subtracted. The binding was assayed 18 hr after the last media change (22 hr after plating) by rinsing cells three times with 1 ml of ice-cold Waymouth's medium containing 30 mM Hepes (pH 7.4) and 0.1% bovine serum albumin. The cells then were incubated with 500 μl of this medium at 4°C for 45 min. Native EGF to determine nonspecific binding was added 30 min before the labeled EGF. Unless otherwise stated, 100 μl of medium containing $^{125}$I-EGF at a final concentration of 0.5 ng/ml (approximately 400,000 CPM) was added to each plate and incubated for 90–120 min at 4°C, a time shown to reach 80–90% of equilibrium in these cells. The medium was removed and the cells rinsed twice with 2 ml Dulbecco's buffered saline with 0.1% BSA at 4°C. For A431 cells, the volume of binding assay was reduced and only 0.1 ng/ml $^{125}$I-EGF was added. Hepatocytes or A431 cells were solubilized on the plate by adding 1% SDS in 0.1N NaOH. After setting covered overnight, the samples were transferred to test tubes and counted using an LKB rackgamma counter. Hepatocyte protein was determined [32] and binding data reported as mean CPM bound/mg protein.

Purified plasma membrane fractions were prepared as previously described [21]. The binding was assessed by incubating 400,000 cpm $^{125}$I-EGF (0.3 ng/ml) with 20 μg membrane protein for 90 min at 21°C in the presence or absence of 1 μg/ml native EGF. Bound $^{125}$I-EGF was separated by retention on membrane filters as described [33]. DMSO was added 5 min prior to the EGF. In some experiments, membrane fractions were pretreated at 21° for 30 min with 3mM TLCK. Under these conditions, EGF-dependent EGF receptor phosphorylation is reduced by 90% (Rubin and Earp, unpublished results).

## RESULTS

Primary cultures of rat hepatocytes were washed and incubated at 0°C in the presence or absence of DMSO. DMSO decreased $^{125}$I-EGF binding in a dose-dependent manner (Fig. 1). In this and other experiments, the reduction was detectable between 1–2% DMSO and was maximal (40–60% reduction) between 5–7.5% DMSO. When the DMSO was removed by washing the monolayers three times prior to the addition of $^{125}$I-EGF, the binding was restored to near control levels. The effect was reversible even if the exposure to DMSO was extended to 90 min before washing and assessment of binding. The onset of the DMSO effect was rapid and could be detected if the solvent was added just prior to the addition of $^{125}$I-EGF (data not shown).

$^{125}$I-EGF binding in whole cells was routinely assessed at 0°C to minimize receptor internalization, but the reduction in binding produced by DMSO was independent of assay temperature. Hepatocytes were incubated for 45 min at 0°C with

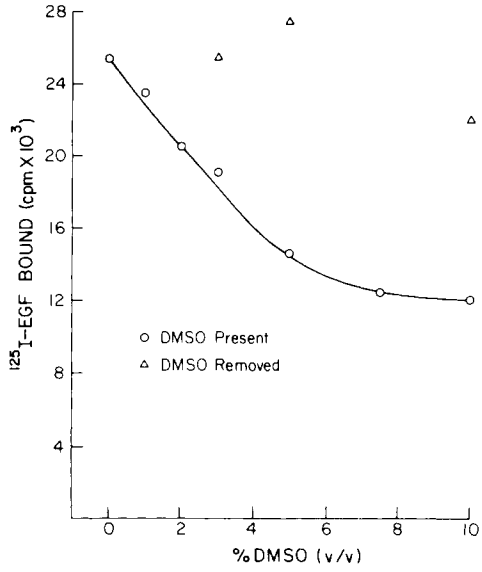


Fig. 1. Effect of DMSO on $^{125}$I-EGF binding to cultured hepatocytes. Hepatocytes were cultured overnight in the presence of 100 nM insulin. DMSO was added 30 min prior to the binding assay at the indicated concentrations. The solvent was either continued or removed prior to the 90 min, 0°C exposure to $^{125}$I-EGF (0.5 ng/ml). Specific binding per mg hepatocyte protein was determined in triplicate.

**TABLE I. The Effect of Assay Temperature on DMSO Reduction in $^{125}$I-EGF Binding***

| DMSO exposure | Cells washed prior to binding | $^{125}$I-EGF binding condition | |
|---|---|---|---|
| | | 0°C, 90 min | 21°C, 30 min |
| None | Yes | 64,678 | 84,482 |
| 0°C, 45 min | Yes | 59,288 | 72,836 |
| 0°C, 45 min | No | 38,567 | 49,151 |

*Hepatocytes were exposed to medium with or without 5% DMSO. DMSO was either removed by washing or continued during the binding assay. Data are the mean of triplicate determinations of specific $^{125}$I-EGF binding (cpm per mg of hepatocyte protein).

DMSO, which then was either removed or continued, followed by addition of $^{125}$I-EGF. A 40–45% reduction in binding was seen with 5% DMSO regardless of whether the binding was assessed at 21°C (30 min) or 0°C (90 min) (Table I). The effect was reversible with washing as measured by either assay protocol (20° or 0°C).

The ability of DMSO to alter EGF binding over a range of EGF receptor concentrations was then tested. Incubation of hepatocytes with glucocorticoid increases EGF binding capacity by 75–100%. Scatchard analysis indicates that the increase is at least in part due to an increase in EGF receptor number [34]. Hepatocytes were maintained for 18 hr in the presence of 100nM insulin or 10 $\mu$M hydrocortisone. The latter doubled the binding. The cells were incubated with various concentrations of DMSO for 30 min at 0°C before adding $^{125}$I-EGF. Hepatocytes cultured with either insulin or hydrocortisone exhibited similar DMSO dose-depen-

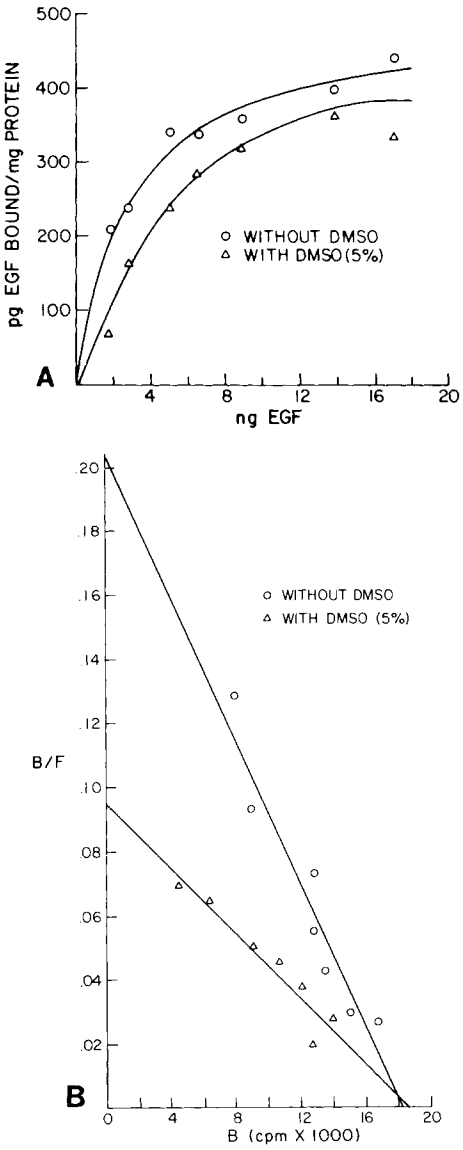


Fig. 2. The effect of DMSO on EGF binding. A population of hepatocytes were incubated with or without DMSO (5%) 30 min prior to the addition of 0.2–3 nM $^{125}$I-EGF for 120 min at 0°C. A) The binding curve. B) Scatchard analysis of the same data. DMSO alters the affinity of the receptor.

dent decreases in EGF binding capacity (data not shown). DMSO also reduced EGF binding in A431 cells, which have approximately 50-fold more EGF receptors than hepatocytes (Table II).

In order to determine whether reduced EGF binding resulted from a decrease in receptor affinity or a functional reduction in receptor number, hepatocytes were incubated with various concentrations of $^{125}$I-EGF in the presence or absence of 5% DMSO. The binding curve (Fig. 2A) and Scatchard plot (Fig. 2B) indicate that EGF

**TABLE II. The Effect of DMSO on $^{125}$I-EGF Binding to A431 Cells***

| | $^{125}$I-EGF Bound | |
|---|---|---|
| %DMSO | DMSO continued | DMSO removed |
| 0 | 31,537 | |
| 1 | 29,680 | 29,504 |
| 2 | 25,793 | 27,688 |
| 3 | 21,300 | 28,013 |
| 5 | 16,996 | 22,685 |
| 7.5 | 15,228 | 22,832 |

*A431 cells were grown to confluence in 24 well trays with 2 $Cm^2$ wells. DMSO at the indicated concentration was incubated with the cells for 30 min prior to assay. All cells were washed and the DMSO either added back (DMSO continued) or not (DMSO removed). The mean of triplicate determinations of specific $^{125}$I-EGF bound is presented. (72,000 cpm, 0.1 ng/ml $^{125}$I-EGF per well.)

**TABLE III. Effect of Solvents on $^{125}$I-EGF Binding in Hepatocytes***

| Solvent | Wash before binding assay | $^{125}$I-EGF bound | % Control | Significance |
|---|---|---|---|---|
| None | Yes | 24,500 | | |
| DMSO | Yes | 27,370 | 62.3 | $p<0.01$ |
| | No | 16,840 | | |
| Ethanol | Yes | 27,330 | 97.7 | N S |
| | No | 26,075 | | |
| Acetone | Yes | 24,520 | 131.1 | N S |
| | No | 31,550 | | |

*The cumulative data from four separate experiments are shown. In each experiment a population of hepatocytes was plated and maintained in 100nM insulin for 18 hr. Medium with or without the indicated solvent (7.5% v/v) was added for 30 min to four plates (triplicate determination of total binding and one for determination of nonspecific binding). One set of solvent treated cells were washed with three changes of plain medium and $^{125}$I-EGF (0.5 ng/ml) was added for 90 min at 0° to all plates. Specific binding was calculated and the mean of the four separate experiments expressed as cpm $^{125}$I-EGF/mg hepatocyte protein. The mean percent control (solvent/solvent removed) is shown also. Only DMSO resulted in a significant decrease in $^{125}$I-EGF binding. The binding of the cells in which the solvent was removed did not differ significantly from that of cells that were never exposed to solvent.

receptor affinity ($K_D$) was reduced in the presence of DMSO from $6.6 \times 10^{-10}$ to $1.5 \times 10^{-9}$M. Receptor number was virtually unchanged.

The time course of association of $^{125}$I-EGF in the presence or absence of DMSO (7.5%) was then studied (Fig. 3). The rate of association of EGF in the presence of DMSO was slower at all times tested (10–150 min).

Two other polar solvents, acetone and ethanol, at concentration of 20%, stimulate EGF receptor phosphorylation in isolated hepatic membranes [21] but are 50% less effective than DMSO. It was of interest, therefore, to see whether these two solvents would also alter EGF receptor binding in live cells. Neither acetone nor ethanol in concentrations equivalent to the maximally effective DMSO concentration reduced EGF receptor binding (Table III).

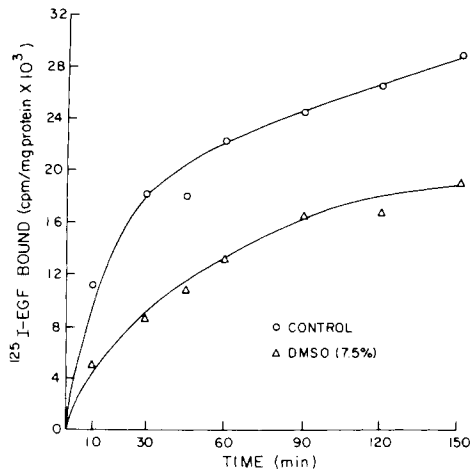


Fig. 3. Time course of $^{125}$I-EGF binding in the presence and absence of DMSO. Hepatocytes were incubated for 30 min (0°C) with and without DMSO prior to the addition of 0.5 ng/ml $^{125}$I-EGF. The binding reaction was stopped at the indicated times.

**TABLE IV. Effect of DMSO on Specific $^{125}$I-EGF Binding in Rat Liver Plasma Membrane***

| | $^{125}$I-EGF bound | |
|---|---|---|
| %DMSO | −TLCK | +TLCK |
| 0 | 33,607 | 37,810 |
| 1 | 30,342 | 33,055 |
| 3 | 26,321 | 28,300 |
| 6 | 18,899 | 21,255 |
| 10 | 13,259 | 17,203 |

*Rat liver plasma membranes (20 μg protein) were incubated with 3 ng/ml $^{125}$I-EGF (400,000 cpm) for 90 min at 21°C with the indicated concentration of DMSO added 5 min prior to the EGF. One set of membranes was preincubated with 3 mM TLCK for 20 min at 21°C prior to the binding assay.

Because DMSO stimulates EGF receptor autophosphorylation, this study determined whether the actual transfer of $P_i$ to the receptor molecule was necessary to change receptor affinity. Purified plasma membranes were incubated with or without DMSO for 5 min prior to the addition at 0.5 nM $^{125}$I-EGF. A dose-dependent reduction in binding occurred in the absence of added ATP (Table IV). A similar reduction was observed when the membranes were pretreated with TLCK at a concentration that reduces the tyrosine kinase activity by greater than 90% (Table IV). Thus, DMSO apparently needs only to effect the conformational change in the receptor to reduce affinity; subsequent autophosphorylation is not required.

## DISCUSSION

The affinity of the EGF receptor is reduced by a rapid action of DMSO that can be reversed at times up to 90 min. Variation of the assay conditions does not alter the result, nor do changes in receptor concentration or cell type. At least two interpretations must be considered: (1) DMSO-dependent conformational change of the EGF receptor results in a decrease in the affinity for EGF. This may be the same alteration in tertiary structure by which DMSO stimulates EGF receptor tyrosine phosphorylation. (2) The solvent may reduce binding by interacting with EGF or the ligand-EGF receptor interface.

Van Obberghen et al reported that DMSO reduced insulin binding in a human lymphocyte line, IM-9 [12]. Their findings were similar to those of this study in that the effect of DMSO was reversible and was due principally to a shift in receptor affinity. The most striking difference was that 10% DMSO almost totally abolished insulin binding. This is not the case with $^{125}$I-EGF; 40–50% residual binding always was observed. These authors suggested that DMSO may change the polar domains of the insulin molecule (or receptor) required for binding.

The techniques used in this report cannot differentiate firmly between the two explanations offered above (change in conformation throughout the receptor or a change limited to the polar interacting groups between the ligand and receptor). Like EGF, insulin stimulates insulin receptor tyrosine phosphorylations [27]. It is interesting to speculate that the alteration of the receptor that results in the kinase-active conformation reduces the affinity of both EGF and insulin receptors to a certain extent. The total abolition of insulin binding by DMSO may indicate that a second process (alteration of ligand tertiary structure) can also occur and is more pronounced at 10% DMSO in the case of insulin. In addition, if the only effect of polar solvents were on the extracellular ligand-receptor interaction (alternative 2), acetone and ethanol would have reduced binding to some extent.

In membranes, acetone and ethanol at 20% concentrations are approximately 50% as effective as DMSO in stimulating EGF receptor phosphorylation (Rubin and Earp, unpublished results). It seems likely that their failure to reduce binding in intact hepatocytes (Table III) is secondary to an inability to gain access to the receptor in situ. The ability of DMSO to penetrate membranes is well known and is probably responsible for a number of its effects, including promoting cellular entry of various pharmacologic agents [1].

Previous findings indicate that DMSO does alter the receptor's cytoplasmic kinase domain. DMSO stimulates EGF receptor phosphorylation in detergent solubilized preparations. In addition, DMSO stimulates receptor phosphorylation when EGF binding is blocked by concanavalin A. Under these conditions, EGF-dependent phosphorylation of the receptor does not occur [21]. Phosphorylation studies also suggest that DMSO affects EGF binding in solubilized receptor preparations. Solubilization enhances EGF-dependent EGF receptor phosphorylation so that up to a 50-fold stimulation is observed. The effect of DMSO is not enhanced in solubilized preparations, and only a six- to sevenfold activation is seen. When EGF and DMSO are combined at optimal concentrations, only a sixfold activation is seen, indicating that DMSO blocks the EGF effect.

Could changes in membrane phosphorylation be one mechanism whereby DMSO alters cell physiology? In membrane fractions, EGF receptor phosphorylation is minimally stimulated at 3% DMSO; the maximal effect occurs at 15–20% [7]. The

binding data indicate that in cells DMSO is effective at lower concentrations, with a 10–25% reduction in EGF binding at 1–3% DMSO. EGF-dependent EGF receptor phosphorylation also exhibits a left shift in the dose-response curve when membranes and whole cells are compared. For example, maximal EGF receptor tyrosine phosphorylation in A431 and liver membranes requires 200–1000 ng/ml EGF [23,33]. When one examines the effect of EGF on phosphotyrosine accumulation in intact A431 cells, as Hunter and Cooper have done, the maximal effect is seen at 50 ng/ml [35]. Further experiments will be necessary to see if 1–3% DMSO will stimulate tyrosine phosphorylation in intact cells.

The data raise the possibility that DMSO may mimic some of the rapid effects of EGF that may be secondary to receptor-kinase activation. In fact, DMSO and EGF share some rapid actions, such as alteration in cell shape [8–10,36] and inhibition of gastric acid secretion [37,38]. Other effects of EGF, such as stimulation of DNA synthesis, requires a longer exposure to EGF [39,40]. DMSO does stimulate DNA synthesis in Novikoff hepatoma cells [41] and in the skin of hairless mice [42]. Whether DMSO stimulates DNA synthesis through an EGF receptor-dependent mechanism is not known. It could be argued that by changing the conformation of the receptor and decreasing the affinity of the receptor for EGF, DMSO may in fact inhibit some steps in EGF action. Therefore, DMSO might stimulate EGF-dependent processes that occur rapidly (perhaps those mediated by tyrosine phosphorylation) but could inhibit the more complex time-dependent actions of EGF.

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