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Oxidative stress induced by L-buthionine-(*S*,*R*)-sulfoximine, a selective inhibitor of glutathione metabolism, abrogates mouse kidney mineralocorticoid receptor function

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Abstract

In vitro studies have demonstrated that cysteine groups present in most of the steroid receptors play an essential role in the steroid binding process as well as in the ability of this superfamily of signaling proteins to function as transcription factors. However, there is poor experimental evidence, if any, which demonstrates that under conditions of oxidative stress the steroid receptors in general, and the mineralocorticoid receptor in particular, are affected in vivo in a similar fashion as has been described for cell-free systems or cells in culture. In the present work we report that when mice are exposed to oxidative stress by treatment with L-buthionine-(*S*,*R*)-sulfoximine (L-(*S*,*R*)-BSO), a glutathione depleting agent, the aldosterone-dependent mineralocorticoid biological response (measured as sodium retention and potassium elimination) was diminished in a directly proportional manner with respect to the depletion of renal glutathione. Accordingly, the steroid binding capacity of the mineralocorticoid receptor was also abrogated, whereas the receptor protein level remained unchanged. The harmful effects observed in mice after glutathione depletion were efficiently prevented by co-treatment with glutathione monoethyl ester. Similar inhibition in the steroid binding capacity was also generated in vitro by receptor alkylation and receptor oxidation, an effect which was prevented in the presence of reducing agents. Since the glutathione deficit generated in vivo by treatment with L-(*S*,*R*)-BSO did not significantly affect other renal proteins which are known to be required for the mineralocorticoid mechanism of action, we suggest that in renal cells a low redox potential exerts drastic and uncompensated inhibition of the receptor-mediated mineralocorticoid biological response. This effect was ascribed to the loss of steroid binding capacity of oxidized receptor, most likely by modification of essential cysteines as supported by experiments where a decreased number of reactive thiols and reduced covalent binding of thiol-reactive ligand were evidenced on immunopurified receptor after in vivo treatment with L-(*S*,*R*)-BSO .

Keywords: Mineralocorticoid receptor; Aldosterone; Sodium retention; Oxidative stress; Glutathione; L-Buthionine-(*S*,*R*)-sulfoximine

Abbreviations: MR, mineralocorticoid receptor; hMR, human MR; GR, glucocorticoid receptor; ALDO, aldosterone; GSH, glutathione; L-(*S*,*R*)-BSO, L-buthionine-(*S*,*R*)-sulfoximine; L-(*R*)-BSO, inactive diastereoisomer of L-(*S*,*R*)-BSO; DTT, dithiothreitol; DTNB, dithionitrobenzoic acid; NEM, *N*-ethylmaleimide; DEM, diethylmaleate; DEX, dexamethasone

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1. Introduction

The mineralocorticoid receptor (MR) is a member of a superfamily of closely related intracellular receptors which function as ligand-activated transcription factors [1]. Aldosterone (ALDO) and 11-deoxycorticosterone are the most potent physiological steroids with mineralocorticoid properties, whereas glucocorticoids such as cortisol and corticosterone are also capable of triggering a MR-dependent biological response under certain conditions. Upon steroid binding, corticosteroid receptors transform and translocate into the nucleus of the target cell in a process where the dissociation of the 90-kDa heat shock protein, hsp90, derepresses the transcriptional activity of the receptor. The temporal sequence for receptor transformation and translocation upon steroid binding is unknown, and the heuristic dogma that hsp90 should be dissociated from the receptor before receptor trafficking into the nucleus is under revision [2–4]. Once the chaperone complex is dissociated, the DNA binding domain of the receptor can bind to cognate responsive elements in the nucleus triggering the specific biological response.

In epithelial cells, ALDO is the main agent responsible for the regulation of the homeostasis of the internal medium by affecting the electrolyte balance through the activation of the MR. Some other factors can also affect sodium elimination via their specific receptors, i.e. arginine vasopressin [5] or atrial natriuretic peptides [6]. Nevertheless, the contribution of ALDO in the maintenance of the electrolyte balance is the most significant. The mineralocorticoid effect is characterized by significant sodium – and water – retention as well as potassium and proton elimination. From the quantitative point of view, the ALDO-dependent sodium retaining effect is much more important than urine acidification or kaliuresis (see [7] for a review and results shown in this work).

As previously noted, endogenous pregnanesteroids with typical glucocorticoid properties (i.e. cortisol and corticosterone) also possess potential mineralocorticoid biological activity. But under physiological conditions these ligands are excluded from competition for the MR due to the inactivating action of the enzyme 11β-hydroxysteroid dehydrogenase that oxidizes the C_{11}-hydroxyl group to 11-keto inactive derivatives [8]. Since ALDO compromises its C_{11}-hydroxyl group in a hemiketalic bridge, it is not a substrate of 11β-hydroxysteroid dehydrogenase. Certain physiological derivatives of progesterone such as 11β-hydroxyprogesterone and 11-ketoprogesterone inhibit the metabolizing activity of 11β-hydroxysteroid dehydrogenase, so that glucocorticoids acquire mineralocorticoid properties. Therefore, a regulatory role for these progesterone derivatives has been postulated for the maintenance of the electrolyte balance [9]. In all the aforementioned instances, the steroids exert their side effects through the activation of the mineralocorticoid receptor, this protein being a key factor for the biochemical events which lead to the maintenance of the electrolyte balance.

Kidney MR is a phosphoprotein [10,11] which is extremely unstable in cell-free systems. Renal MR is particularly sensitive to oxidants in a low potential redox medium [12]. According to this feature and due to the inhibitory effect of alkylating reagents on steroid binding, an essential role for cysteines was inferred for both rat [12,13] and human MR (hMR) [14]. This inference was confirmed in a recent work by site-directed mutagenesis of Cys^{849} and Cys^{942} of the hMR [15].

Both the glucocorticoid receptor (GR) and the MR lose their ability to bind steroid upon in vitro treatment with sulfhydryl reagents. Interestingly, native MR seems to be extremely more sensitive to this inactivation than native GR since a concentration as low as 10 μM of sulfhydryl reagent is sufficient to abolish one-third of the steroid binding capacity of the MR. In contrast, at least a 10-fold higher concentration of thiol reactive reagent is required to obtain similar inhibition with the GR. Accordingly, renal MR is highly sensitive to inactivation by metal ions (both in vitro and in vivo), particularly those that belong to the hard ion type (i.e. Hg^{2+}, Cd^{2+}, Cu^{2+}) which are highly reactive with thiol groups in agreement with the soft and hard theory for metal ion reactivity [13].

Preserving protein thiols in a reduced form is an important challenge for the cell. As a defense against oxidative metabolism, cells possess the ability to synthesize quenchers that intervene either as sacrificial molecules in redox cycles or as specific enzymes that act directly on the oxygen radicals forming products that are less offensive. There are at least three major antioxidant enzymatic systems: superoxide dismu-

tase, catalase, and glutathione peroxidase [16]. Superoxide dismutases convert superoxide anion into H_2O_2 and O_2, catalase catalyzes the dismutation of H_2O_2, and glutathione peroxidase catalyzes the reductive destruction of oxidized compounds using reduced glutathione as an electron donor.

Glutathione (GSH) is a tripeptide (γ-Glu-CysH-Gly) widely distributed in animals, plants and microorganisms [17,18]. It is typically present in high levels (mM) and is thus both the most prevalent intracellular thiol and the most abundant low molecular weight oligopeptide. GSH is not required in the diet, but is synthesized in virtually all animal cells by the sequential actions of two enzymes: γ-glutamyl-cysteine synthetase and GSH synthetase. GSH is exported continuously from the cell and its degradation occurs extracellularly [19]. GSH deficiency can be produced in vivo by administering animals a transition state inhibitor of γ-glutamylcysteine synthetase, L-buthionine-(*S*,*R*)-sulfoximine (L-(*S*,*R*)-BSO) [20,21]. Therefore, cellular GSH level decrease after L-(*S*,*R*)-BSO is given because the cellular export of GSH continues in the absence of significant intracellular synthesis [18].

The requirement of a low redox potential for steroid binding to the MR has been previously studied in vitro, mostly in structural studies which ascribed the inactivation of the MR to the crucial role played by certain amino acids in the receptor, most likely cysteine. Whether the inactivation of MR also occurs in vivo under circumstances of oxidative stress is uncertain. In the present work, we report that when renal GSH is depleted by treatment of mice with L-(*S*,*R*)-BSO, both the mineralocorticoid biological response and the ALDO binding capacity of kidney MR are abrogated. We also provide direct evidence that the inhibition of these two processes is correlated with the oxidation of thiol groups of the MR.

2. Materials and methods

2.1. Reagents

[1,2-^{3}H]ALDO (50.0 Ci/mmol), [6,7-^{3}H]dexamethasone (DEX) (40.5 Ci/mmol), [6,7-^{3}H]DEX-mesylate (35.5 Ci/mmol), and radioinert ZK91587 were purchased from New England Nuclear (Boston, MA, USA). [21,22-^{3}H]Ouabain (35.0 Ci/mmol) was from Amersham (Arlington Heights, IL, USA). [^{14}C]Amiloride was synthesized with [^{14}C]methyl iodide (Amersham, 28 Ci/mmol) and purified by thin layer chromatography as described by Lazorick et al. [22]. Radioinert ALDO, DEX, L-(*S*,*R*)-BSO, reduced GSH, GSH monoethyl ester, dithionitrobenzoic acid (DTNB), *N*-ethylmaleimide (NEM), diethylmaleate (DEM), and amiloride were from Sigma Chemical Co. (St. Louis, MO, USA). RU28362 and RU486 were a kind gift from Roussell-Uclaf (Romainville, France). The rabbit pAbhMR antibody generated against the hMR amino acids 1–719 was a generous gift of Dr. G. Litwack and Dr. N. Robertson from the Jefferson Cancer Institute (Philadelphia, PA, USA). Donkey anti-rabbit IgG-horseradish peroxidase was from Pierce (Rockford, IL, USA). L-(*S*,*R*)-BSO was recrystallized from 80% (v/v) ethanol and fractionally crystallized from water to obtain the less soluble diastereoisomer L-(*R*)-BSO as described by Mårtensson et al. [21].

2.2. Determination of mineralocorticoid activity

Bioassays were always carried out according to the ethical requirements for animal handling ruled by national authority. Male BALB/c mice weighing ~15 g underwent adrenalectomy 5 days prior to the experiments. Mice were fed Purina Diet 1 and 0.9% sodium chloride and fresh water ad libitum. Saline solution also contained 50 μg/ml of DEX. This saline/DEX solution was replaced by saline solution without steroid the night before the experiment. Food was also removed at this time. All liquids were removed 4 h before ALDO was administered. For the bioassays, we followed a method previously described in detail for rats [23,24]. Briefly, mice were anesthetized with ether and their urinary bladders were emptied by suprapubic pressure. The penis was ligated to favor urine retention and mice were given intramuscular injections of ALDO dissolved in ethanol:propylene glycol:0.9% NaCl solution (1:2:37) at a dose equal to 2 μg steroid per 100 g of body weight for most of the experiments. Controls were injected with vehicle only. A subcutaneous injection of 3 ml of 0.9% NaCl solution was also given. Three hours later, mice were anesthetized again and

urine was aspirated from the bladder. Urinary sodium and potassium levels were measured by flame photometry. Systematically, plasma and urinary creatinine were also measured to evaluate the endogenous creatinine clearance rate.

2.3. In vitro steroid binding assays

Kidneys from adrenalectomized mice were excised after extensive perfusion with ice-cold phosphate saline solution. Renal cortex–medulla interphases were homogenized in two volumes of a buffer containing 25 mM Tris, 10 mM EDTA, 20 mM sodium molybdate, 20% glycerol, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, 2 IU/ml aprotinin, 30 μg/ml trypsin-chymotrypsin inhibitor, at pH 7.4. The homogenates were centrifuged at $67\,000\times g$ for 45 min at 0°C, and the supernatant from this centrifugation was referred to as the cytosol. Kidney cytosol was incubated for 10 h at 0°C in the presence of 10 nM [^{3}H]ALDO and 1.0 μM RU28362 to prevent cross-reaction with the GR. A 1000-fold excess of radioinert ALDO was used to determine the non-specific binding (20–30% of the total). The standard reaction volume used for these studies was 0.5 ml. Bound steroid was separated from free by adding 1 volume of 2% charcoal coated with 0.2% dextran 15–20. Scatchard plots were performed by incubation of kidney cytosol with increasing concentrations of [^{3}H]ALDO (from 1×10^{-10} M to 5×10^{-8} M) as described in previous works [24,25].

Steroid binding to the GR was measured in cytosols incubated with 50 nM of [^{3}H]DEX in the presence of 10.0 μM ZK91587 to prevent cross-reaction with the MR. The non-specific binding was measured in the presence of 1000-fold of radioinert DEX (10–20% of the total).

2.4. Sulfhydryl modification in kidney cytosol

Kidney cytosol was obtained from adrenalectomized mice in buffer lacking DTT. The MR was inactivated by alkylation with 0.1 mM or 1.0 mM of NEM or DTNB for 1 h at 8°C. MR oxidation was induced with 5% (v/v) H_2O_2 for 30 min at 8°C, in the presence of 1 mM NaN_3 to inhibit endogenous catalases. A steroid binding assay with 10 nM [^{3}H]ALDO and 1.0 μM RU28362 was then performed. When the reversion of thiol blocking was attempted, a preincubation of 30 min on ice with 10 mM DTT was conducted prior to the steroid binding assay, which was also performed in a medium containing DTT.

2.5. Depletion of glutathione

L-(*S*,*R*)-BSO and L-(*R*)-BSO were dissolved in ethanol:propylene glycol:0.9% NaCl solution (3:5:32). Intramuscular injections were given to adrenalectomized mice at intervals of 12 h (8.00 and 20.00 h) during 5 days (2.5 mmol BSO/kg body weight at each injection or 5 mmol/kg/day). In treatments where GSH or GSH monoethyl ester were also injected, three daily doses of 2 mmol/kg per injection were also given (9.00, 15.00, and 21.00 h). Acute treatments with DEM (dissolved in the aforedescribed vehicle) were conducted by injection of 3 mmol/kg every 60 min for a total period of 2 h (three doses). After the third dose, the assays to evaluate the mineralocorticoid response were performed.

2.6. Glutathione determinations

Blood samples were taken by heart puncture and collected in 5 mM EDTA. Plasma was separated quickly, acidified with 1% (w/v) 5′-sulfosalicylic acid to prevent autooxidation of GSH, centrifuged at $1000\times g$ for 5 min, and plasma was used to measure the GSH concentration. When GSH levels were measured in other tissues, the organs were perfused with ice-cold saline solution, excised, and homogenized in 5 volumes of 5% (w/v) 5′-sulfosalicylic acid. The homogenates were centrifuged at $8100\times g$ for 10 min and the supernatants were used to measure GSH levels. Total GSH was measured by the GSH disulfide reductase–DTNB recycling enzymatic assay as described by Anderson [26].

2.7. In vivo steroid binding assays

Three groups of adrenalectomized mice were treated for 5 days (long-term treatment) with either L-(*S*,*R*)-BSO, L-(*R*)-BSO or vehicle. A fourth group was treated with a single dose of 5 mmol L-(*S*,*R*)-BSO/kg (acute treatment). We used a modification of a previously described assay for in vivo ALDO

binding to kidneys [23]. Animals were given intraperitoneal injections of 10 μCi [^{3}H]ALDO and 20 μg of RU28362 in saline. After acute treatment, mice were injected with [^{3}H]ALDO 5 h after the injection with L-(*S*,*R*)-BSO. Kidneys were excised 20 min after the radioactivity was injected and homogenized in the same buffer described above for the in vitro assays, but without the addition of DTT. Kidney cytosol was adsorbed with charcoal/dextran to clarify free [^{3}H]ALDO, and the remaining radioactivity was measured in the supernatant. The non-specific binding (~50% of the total binding) was measured by co-injection of 30 μg of radioinert ALDO and subtracted from the total. In some experiments, in vivo labelled cytosol was reincubated in vitro with 10 nM [^{3}H]ALDO and 1.0 μM RU28362 in the presence of 2 mM DTT.

2.8. Immunoprecipitation of renal MR

To avoid the slight cross-reactivity of pAbhMR antibody with the GR, renal cytosol was precleared with BuGR2 antibody for 1 h at 4°C as described in detail [11]. The resultant supernatant was immunoadsorbed with pAbhMR antibody against MR (or non-immune rabbit serum) bound to protein A-Sepharose for 2 h at 4°C, and washed five times with ice-cold buffer containing 10 mM Tris, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.5 M NaCl, at pH 7.5. Proteins were resolved in a 9% polyacrylamide-SDS gel, electrotransferred to an Immobilon membrane, and probed with a solution of 0.1% pAbhMR. Donkey anti-rabbit antibody labelled with either ^{125}I or horseradish peroxidase was used to visualize immunoreactive bands.

When sulfhydryl groups of the MR were quantified, immunopellets obtained as described above were stripped of proteins associated with the receptor by incubation with buffer containing 0.5 M NaCl (1 h at 0°C). Stripped immunopellets were washed three times with 80 mM phosphate buffer containing 1 mM EDTA (pH 8.0). Total protein sulfhydryl was measured as described by Habeeb [27] with Ellman's reagent (made in the same buffer used to wash the pellets) supplemented with 2% SDS.

2.9. Receptor labelling with [^{3}H]DEX-mesylate

Mice were treated with L-(*S*,*R*)-BSO for 5 days as described above. Kidneys were excised and homogenized as described for binding assays, except that DTT was omitted from the buffer. Cytosol was labelled for 3 h at 0°C with 300 nM of [^{3}H]DEX-mesylate in the absence (total binding) or presence (non-specific binding) of 50 μM radioinert DEX. To avoid labelling of the GR, the incubation medium was supplemented with 30 μM RU486. Free steroid was adsorbed with 1% charcoal/0.1% dextran, and the resultant labelled MR was immunoadsorbed and resolved by electrophoresis as described above.

2.10. Isolation of kidney tubules

Distal kidney tubules were obtained according to

Table 1
GSH levels in mouse tissues after treatment with L-(*S*,*R*)-BSO

Tissue	GSH, μmol/g of tissue (% of control)		
	Control	L-(*S*,*R*)-BSO	L-(*R*)-BSO
Plasma[a]	20.2 ± 0.8	4.7 ± 0.8 (23.2)	17.5 ± 0.5 (86.6)
Kidney	2.2 ± 0.2	0.4 ± 0.1 (18.2)	1.8 ± 0.2 (81.2)
Hippocampus	1.6 ± 0.3	1.0 ± 0.2 (62.5)	1.4 ± 0.3 (87.5)
Parotid	1.7 ± 0.4	0.6 ± 0.3 (35.3)	1.5 ± 0.2 (88.2)
Distal Colon	2.1 ± 0.4	0.5 ± 0.2 (23.8)	1.8 ± 0.3 (85.7)
Heart	2.3 ± 0.2	0.4 ± 0.1 (17.4)	2.0 ± 0.2 (87.0)
Liver	6.5 ± 0.5	2.2 ± 0.3 (33.8)	5.1 ± 0.3 (78.5)
Lung	1.9 ± 0.2	0.6 ± 0.2 (31.6)	1.6 ± 0.2 (84.2)

Adrenalectomized mice were injected (i.m.) for 5 days at intervals of 12 h with either 5.0 mmol/kg/day of L-(*S*,*R*)-BSO, L-(*R*)-BSO, or vehicle (control). Organs were excised, homogenized, and GSH levels measured. Results represent means ± S.E.M. for five mice per group.

[a]Plasma concentrations are given in μM.

Gesek et al. [28]. Briefly, mouse kidneys were perfused with Krebs–Henseleit solution containing 1100 U/ml of collagenase and 400 U/ml hyaluronidase, excised, and cut into sagittal slices of ~2 mm thick. Cortices were carefully removed, minced, and incubated with collagenase and hyaluronidase for 15 min at 37°C with continuous shaking (100 rpm) under a 95% O_2/5% CO_2 atmosphere. The suspension of dispersed tubules was separated on Percoll isoosmotic gradient (previously bubbled with 95% O_2) for 10 min at 17 500×*g* (4°C). Bands enriched in distal tubules (density 1.020–1.025 g/ml) were pooled and immediately examined under the microscope. Isolated tubules were finally placed in Dulbecco's modified Eagle's medium supplemented with 10% mouse serum. Viability was estimated by both trypan blue exclusion and lactate dehydrogenase leakage. Distal tubules were used to evaluate several parameters according to methods previously described in the literature: 11β-hydroxysteroid dehydrogenase activity as described [9], [^{14}C]amiloride binding capacity according to Sariban-Sohraby et al. [29], and citrate synthase activity, Na/K-ATPase activity, and [^{3}H]ouabain binding capacity as described by Laplace et al. [30].

3. Results

3.1. Treatment with L-(S,R)-BSO generates a systemic deficiency of GSH

Table 1 shows GSH levels in tissue preparations obtained from several organs. Except liver, all organs are targets for ALDO. Administration of L-(*S*,*R*)-BSO led to decreasing GSH levels, whereas the inactive diastereoisomer L-(*R*)-BSO was ineffective in depleting GSH as compared with untreated controls.

The concentration of GSH ranged from ~18% of the control level for the most sensitive tissues (kidney and heart), to ~35% for the other studied tissues (plasma, parotid gland, distal colon, liver, and

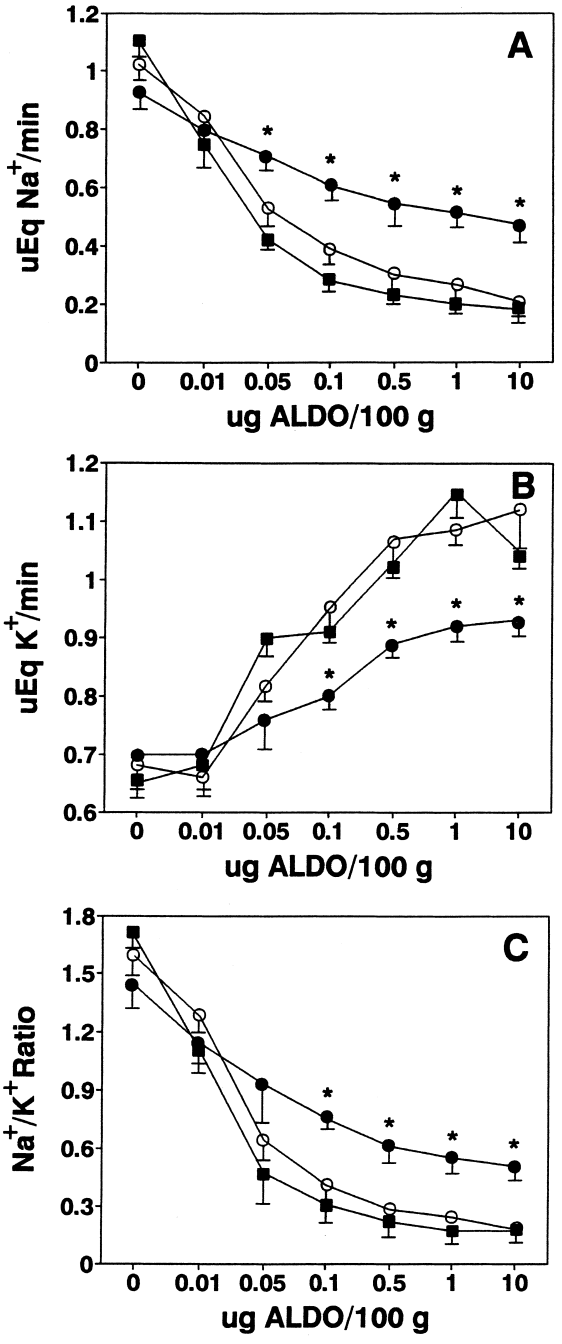

Fig. 1. Mineralocorticoid biological effect in mice treated with buthionine-sulfoximine. Adrenalectomized mice were treated for 5 days with 5.0 mmol/kg/day (administered in two injections every 12 h) of either L-(*S*,*R*)-BSO (●) or the inactive diastereoisomer L-(*R*)-BSO (○). Vehicle alone was administered to controls (■). Different doses of ALDO were then administered (i.m.) as described in Section 2, urine was aspirated after 3 h by bladder puncture, and sodium, potassium, and creatinine were measured. Results show urinary elimination of electrolyte as a function of the logarithm of the injected dose of ALDO (mean ± S.E.M. of four experiments, each performed with four animals per dose and per experimental condition). *Different from controls at $P < 0.001$.

lung). Levels of GSH in the hippocampus decreased only 38%, probably as a consequence of the relatively poor diffusion of L-(*S*,*R*)-BSO across the blood–brain barrier. Nevertheless, GSH levels are significantly lower in the hippocampus from L-(*S*,*R*)-BSO-treated animals than in untreated mice.

3.2. The mineralocorticoid function is inhibited in mice treated with L-(S,R)-BSO

Fig. 1 depicts dose–response curves for the mineralocorticoid effect on adrenalectomized mice injected with several doses of ALDO. In control mice, the rate of sodium retention reached a plateau at 1 μg/100 g dose. Consistent with the major role of ALDO in controlling the electrolyte balance, only 16% of sodium elimination with respect to mice injected with vehicle was measured at that saturating dose (Fig. 1A). As previously noted, the kaliuretic effect is less sensitive than the natriuretic effect, and nearly 70% of increasing potassium elimination was measured (Fig. 1B). Therefore the Na^+/K^+ ratio, a more representative parameter of the mineralocorticoid effect, diminished 10-fold after treatment with agonist (Fig. 1C).

Mice pretreated with 5 mmol/kg/day of L-(*S*,*R*)-BSO, a selective inhibitor of GSH metabolism, exhibited a significantly ($P < 0.001$) less efficient mineralocorticoid effect upon ALDO administration. As can be seen in Fig. 1A, untreated mice show ~ 2.5 times more sodium retention at doses ≥ 0.1 μg ALDO. Accordingly, the ALDO-dependent kaliuresis is $\sim 30\%$ less effective ($P < 0.001$). As a consequence, the Na^+/K^+ ratio is about three times higher than the values exhibited by controls (Fig. 1C). In contrast, animals treated with the inactive diastereoisomer *L*-(*R*)-BSO showed no significant difference for both electrolytes with respect to the control group.

Importantly, the endogenous creatinine clearance rate remained constant (0.11 ± 0.04 ml/min) regardless of the treatment, demonstrating that if a diminished electrolyte elimination rate was measured, it was due to the effect of the steroid on the target cell and not to changes in the mouse's glomerular filtration rate. Accordingly, the values of Na^+ retention and K^+ elimination were similar for all conditions in animals which were not injected with ALDO.

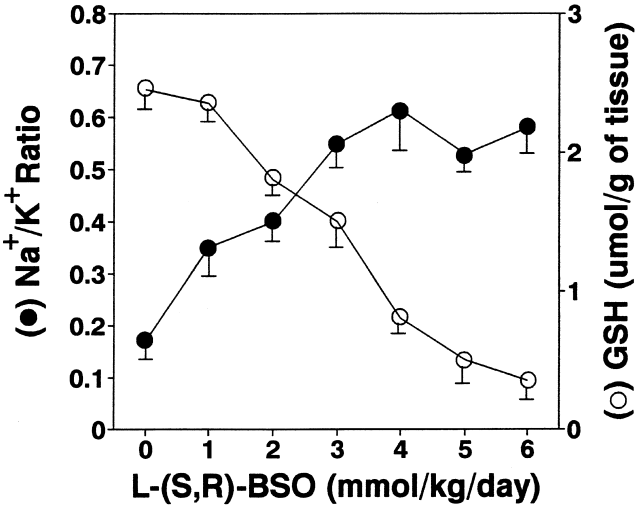

Fig. 2. Dependence of tissue GSH levels and inhibition of the mineralocorticoid effect on the dose of L-(*S*,*R*)-BSO. Daily total doses of L-(*S*,*R*)-BSO ranging from 0 to 6 mmol/kg were given to mice for 5 days in two injections every 12 h. 2 μg of ALDO per 100 g of body weight was then injected and the mineralocorticoid effect measured as described for Fig. 1. After collecting urine samples, kidneys were quickly removed, homogenized, and the GSH tissue levels quantified. Results show the mean ± S.E.M. of eight animals per treatment.

3.3. The inhibition of the mineralocorticoid effect and the depletion of renal GSH are related in a dose-dependent manner with the administration of L-(S,R)-BSO

The previous experiments demonstrated that treatment with L-(*S*,*R*)-BSO depletes tissue GSH and the ALDO-dependent biological response is partially abolished as well. In order to demonstrate a direct link between these variables, a group of adrenalectomized mice were treated with several doses of L-(*S*,*R*)-BSO for 5 days, and then injected with 2 μg ALDO/100 g to ensure maximum sodium retention and potassium elimination. Once samples of urine were taken by bladder puncture, kidneys were excised and homogenized to determine the tissue GSH concentration.

As can be seen in Fig. 2, renal GSH levels and inhibition of the mineralocorticoid effect show an inverse relationship, suggesting that the low redox potential generated in the tissue by treatment with L-(*S*,*R*)-BSO may be responsible for the lack of a proper biological response upon ALDO stimulation. Importantly, this correlation is dose-dependent with

the GSH depleting agent. Inasmuch as a 5 mmol/kg/day dose exhibits almost maximum effect, and because it was always well tolerated by the mice, we selected this dose for the rest of the experiments shown in this work.

3.4. Effect of acute treatment with L-(S,R)-BSO

The temporal effect of a single intraperitoneal dose of 5 mmol/kg L-(*S*,*R*)-BSO and L-(*R*)-BSO on renal GSH content is shown in Fig. 3A. Maximum depletion of GSH occurred 3 h after drug administration and remained approximately constant for 6 h. Renal GSH level recovered to similar values as those measured in the control after 15 h. On the other hand, the inactive isomer L-(*R*)-BSO exhibited no effect on GSH renal content.

In order to evaluate whether acute depletion of GSH also inhibits the ALDO-dependent mineralocorticoid effect, a group of mice were pretreated with 5 mmol/kg L-(*S*,*R*)-BSO for 5 h and another group were pretreated during 5 days as described for Fig. 1. Sodium and potassium elimination were then measured after injection of a saturating dose of 2 μg/100 g of ALDO. Fig. 3B shows that acute GSH depletion also inhibited the mineralocorticoid biological response in a similar manner as observed for mice treated during 5 days.

Results depicted in Fig. 3 confirm that a diminished redox potential exerts deep effects on the ALDO-mediated mineralocorticoid response in kidney. They also suggest that the consequences of a long-term treatment on this in toto biological response are the same as those observed in acute treatment, making unlikely the influence of putative side effects on the mineralocorticoid response after 5 days of GSH depletion.

3.5. GSH depletion decreases the steroid binding capacity of the renal MR

Because glutathione depletion inhibited the ALDO-dependent biological response, we analyzed the in vivo renal steroid binding capacity of mouse MR after treatment with buthionine sulfoximine. Mice were pretreated with 5 mmol/kg L-(*S*,*R*)-BSO for 5 h or 5 days, and were then injected (i.p.) with 10 μCi of [^{3}H]ALDO and 20 μg of RU28362 in saline solution (±30 μg of radioinert ALDO). Under these conditions, renal radioactivity reaches a maximum after 20 min post-injection (data not shown), so kidneys were excised at this time. Cytosol was obtained in buffer lacking DTT and cleared of free [^{3}H]ALDO by adsorption with charcoal/dextran. Specific binding was measured and the results are depicted in Fig. 4A. Kidney cytosol obtained from

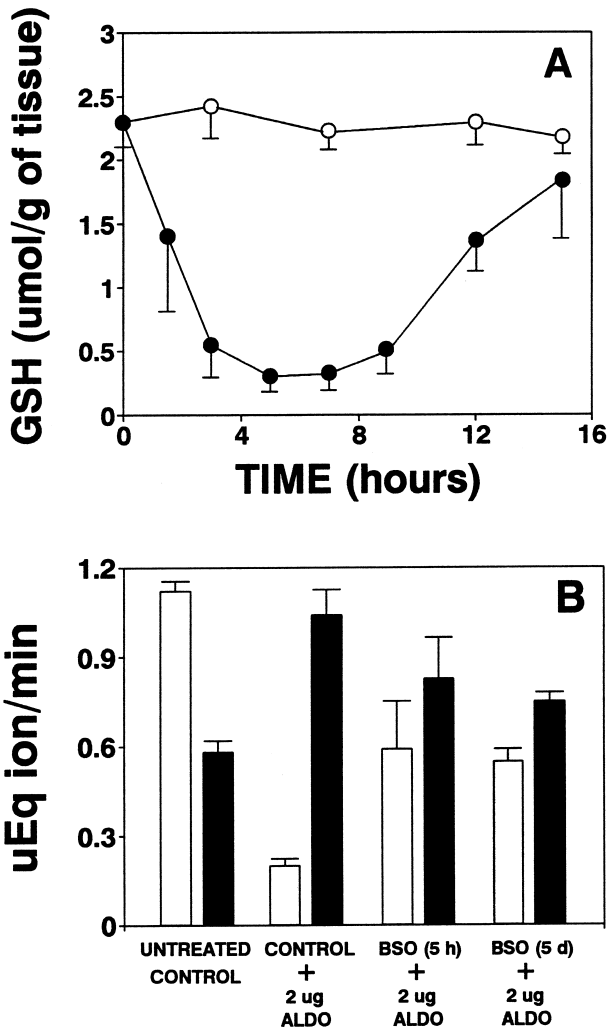

Fig. 3. Acute treatment with L-(*S*,*R*)-BSO. (A) Time course of renal GSH levels after a single dose of L-(*S*,*R*)-BSO. GSH was measured in mouse kidney cytosol after the indicated post-injection times of a single intraperitoneal dose of 5 mmol/kg L-(*S*,*R*)-BSO (●) or 5 mmol/kg L-(*R*)-BSO (○). Results are means ± S.E.M. ($n=5$). (B) Mineralocorticoid effect. Mice were treated with 5 mmol/kg of L-(*S*,*R*)-BSO for 5 h or 5 days. Urinary elimination of sodium (open bars) and potassium (black bars) was measured after injection of a saturating dose of 2 μg/100 g of ALDO. Results are means ± S.E.M. ($n=8$).

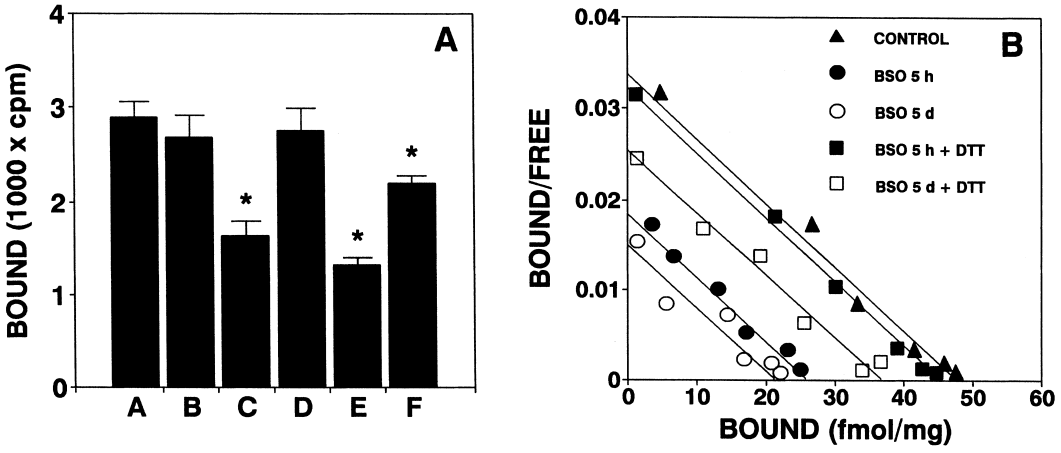

Fig. 4. Steroid binding capacity of renal MR from treated mice. (A) In vivo labelling. Mice were treated with vehicle (column A), L-(*R*)-BSO for 5 days (column B), L-(*S*,*R*)-BSO for 5 h (columns C and D) or for 5 days (columns E and F). Then, an intraperitoneal injection of 10 μCi [^{3}H]ALDO and 20 μg RU28362 (±30 μg radioinert ALDO) was given, kidneys were removed after 20 min and homogenized in buffer lacking DTT. Free [^{3}H]ALDO was clarified by adsorption with charcoal/dextran and the radioactivity was measured in aliquots containing 5 mg of protein. In conditions D and F, the in vivo labelled cytosol was preincubated on ice with 2 mM DTT for 30 min, and then reincubated in vitro with 10 nM [^{3}H]ALDO (±1000-fold of radioinert ALDO) for 3 h at 0°C in the presence of 2 mM DTT. Results are means ± S.E.M. of four animals per group. *Different from condition A at $P < 0.001$. (B) Scatchard plots. Kidney cytosol was obtained from mice treated with 5.0 mmol/kg/day of L-(*S*,*R*)-BSO for 5 days. Cytosol (5 mg of protein) was labelled with increasing concentrations of [^{3}H]ALDO for 3 h at 0°C. Specific binding was then measured. Conditions are: cytosol from untreated mice (▲); cytosol from L-(*S*,*R*)-BSO-treated mice for 5 days (○) or 5 h (●); cytosol from L-(*S*,*R*)-BSO-treated mice for 5 days (□) or 5 h (■) which was incubated with 2 mM DTT on ice for 30 min and relabelled in vitro with [^{3}H]ALDO in the presence of the reducing reagent.

mice pretreated with the inactive isomer L-(*R*)-BSO (column B) showed similar specific binding as the controls treated with vehicle (column A), whereas renal cytosol from animals treated for 5 h with L-(*S*,*R*)-BSO showed nearly 45% lower steroid binding capacity (column C). Radiolabelled cytosols reincubated in vitro with 10 nM [^{3}H]ALDO for 3 h at 0°C showed no increase in the specific radioactivity bound to MR, indicating that the in vivo labelling had saturated the renal MR binding capacity (data not shown). When cytosol obtained from mice treated in acute form with L-(*S*,*R*)-BSO was reincubated in a buffer containing DTT, MR completely recovered the steroid binding capacity to the same levels as control (column D). This suggests that the lack of steroid binding capacity exhibited by the MR in this group of mice was due to a rapid oxidation of MR in a GSH-depleted milieu (0.4 ± 0.2 μmol GSH/g of tissue). This observation also indicates that the receptor protein should be present at normal levels in kidney after the acute treatment with buthionine sulfoximine, although it is unable to bind steroid efficiently. Column E shows a diminished steroid binding capacity of kidney cytosols obtained from mice treated for 5 days with L-(*S*,*R*)-BSO. This value is not significantly different from the acute (5 h) treatment. However, reincubation of cytosol with [^{3}H]ALDO in the presence of DTT failed to recover fully the steroid binding capacity upon reduction of oxidized proteins. Thus, a significant reduction of 25% in the steroid binding was measured. This may be due to a change in the number of receptors able to bind steroid or because the receptor lost affinity for the ligand. Therefore, we performed Scatchard plots to evaluate these options. Fig. 4B shows that control cytosol exhibits a steroid binding capacity of 48 ± 5 fmol/mg of protein whereas only 22 ± 5 and 26 ± 2 fmol/mg of protein were measured, respectively, from 5 days and 5 h L-(*S*,*R*)-BSO-treated mice. When these samples were preincubated on ice with 2 mM DTT for 30 min and the steroid binding assay was then performed, the total number of receptors was fully recovered in cytosol obtained from 5 h treatment (53 ± 3 fmol/mg), whereas only a partial recovery was obtained in cytosol from 5 day treatment (36 ± 3 fmol/mg). The dissociation constants

Table 2
Effect of L-(*S*,*R*)-BSO on the steroid receptor binding capacity of different organs

Tissue	Receptor	Control	+L-(*S*,*R*)-BSO	
			5 h	5 days
Kidney	MR	47 ± 6	28 ± 4[a]	23 ± 3[a]
	GR	140 ± 15	121 ± 7	109 ± 15[d]
Liver	MR	Not detectable	Not detectable	Not detectable
	GR	475 ± 38	377 ± 22[b]	296 ± 31[a]
Lung	MR	16 ± 4	10 ± 1[c]	7 ± 3[c]
	GR	185 ± 21	157 ± 13[a]	112 ± 9[a]
Hippocampus	MR	75 ± 7	68 ± 9	55 ± 6[d]
	GR	105 ± 12	109 ± 7	90 ± 6
Parotid	MR	19 ± 2	12 ± 1[b]	8 ± 2[a]
	GR	47 ± 8	35 ± 6	28 ± 4[a]
Distal colon	MR	30 ± 6	20 ± 5[d]	16 ± 4[b]
	GR	55 ± 7	42 ± 6	35 ± 5[b]

Adrenalectomized mice were treated for 5 h or 5 days with 5 mg/kg of L-(*S*,*R*)-BSO. Controls were injected with vehicle only. Organs were removed, homogenized in buffer lacking DTT, and centrifuged. The specific binding of [^{3}H]ALDO to the MR and [^{3}H]DEX to the GR were measured in cytosolic fractions as described in Section 2. Results represent the mean (in fmol/mg) ± S.E.M. of six animals per group. Significantly different from control at [a] $P < 0.001$, [b] $P < 0.005$, [c] $P < 0.010$, [d] $P < 0.015$.

measured for all conditions were nearly identical and may be clustered around a mean value of 0.6 ± 0.2 nM. Previous experiments performed as control demonstrated that L-(*S*,*R*)-BSO does not itself affect the ALDO binding to MR when the drug is added to the incubation medium in a cell-free system.

These experiments demonstrate that the partial decrease of steroid binding capacity in L-(*S*,*R*)-BSO-treated mice may be attributed to a loss in the number of renal binding sites rather than changes in the affinity constant of MR for ALDO.

3.6. Steroid binding capacity in different organs

We next asked if glutathione depletion is also able to inhibit the MR binding capacity in target organs other than kidney, as well as whether the GR is as sensitive to such depletion as the MR. Mice were treated for 5 h or 5 days with L-(*S*,*R*)-BSO and kidney (control), liver (MR is not expressed in this organ), lung, hippocampus, parotid, and distal colon were excised, homogenized in buffer lacking DTT, and a steroid binding assay was then performed on cytosol fractions. Specific binding to the MR was measured with 10 nM [^{3}H]ALDO (± 1000-fold excess of radioinert ALDO) in the presence of 1.0 μM RU28362 to block the GR, and 50 nM [^{3}H]DEX (± 1000-fold excess of radioinert DEX) was used to evaluate the GR steroid binding capacity. To prevent the cross-reaction of DEX with the MR, 10 μM of the specific antimineralocorticoid ZK91587 was used. Results are shown in Table 2.

As demonstrated before for kidney, treatment with L-(*S*,*R*)-BSO significantly inhibited the ALDO binding to the MR in lung, parotid, and distal colon (approximately 40% and 50% after 5 h and 5 days of treatment, respectively). In contrast, hippocampus exhibited lower sensitivity than the other target organs. In effect, only 26% of inhibition with respect to the control was measured after 5 days of treatment and no significant difference was observed for acute treatment. This observation in hippocampus parallels the lower efficiency of L-(*S*,*R*)-BSO to deplete GSH in this tissue (Table 1). On the other hand, results shown in Table 2 demonstrate that the GR is less sensitive than the MR to the GSH depletion. No significant differences in DEX binding were obtained after 5 h of treatment in kidney, lung, hippocampus, parotid, and colon[1], whereas only 20% inhibition was observed in liver. Long-term treatment decreased

[1] Note that values of steroid binding measured in colon (expressed per mg of protein) are significantly lower than values reported by other groups [42] because we used crude cytosol rather than cytosol from isolated colonic crypts as source of receptors.

by 20% the steroid binding capacity in kidney, whereas ~35% inhibition was measured for the other tissues, except hippocampus. Interestingly, in vitro treatments with DTT (data not shown), as those performed in the experiments shown in Fig. 4, fully reversed the inhibition of the steroid binding capacity of both the GR (in all tissues) and the MR (except in kidney).

Taken together, these results obtained by oxidative stress generated in vivo confirm previous observations performed in vitro which reported a lower sensitivity for GR than for MR in a low redox potential. This also suggests that the MR may be more sensitive to an oxidative environment due to its structural and/or regulatory features rather than a non-specific systemic failure due to the GSH depletion.

3.7. Sulfhydryl-modifying reagents inhibit the steroid binding capacity of MR in a reversible manner

If the oxidation of thiol groups of MR is responsible for the loss of steroid binding capacity, sulfhydryl-reacting reagents should exert similar effects. To confirm this hypothesis, mouse kidney cytosol obtained in buffer lacking DTT was treated with either NEM and DTNB (0.1 mM and 1.0 mM for 1 h at 8°C) to promote thiol alkylation, or with 5% H_2O_2 for 30 min at 8°C to promote MR oxidation. A steroid binding assay was then performed without further treatment or after previous reincubation of thiol-modified cytosol with 10 mM of DTT.

Fig. 5 shows that NEM, DTNB, and H_2O_2 inhibited ALDO binding in a DTT-reversible manner, suggesting a crucial role of reduced cysteines in the MR for steroid binding. These results obtained with mouse MR are consistent with previous observations reported with rat MR [12,13] and recombinant hMR [14]. Importantly, the inhibition obtained in vitro with cysteine-reactive reagents resembles the effects observed in the aforementioned experiments where oxidative stress was generated in vivo by depletion of GSH.

3.8. GSH monoethyl ester reverses the inhibitory effect of L-(S,R)-BSO on the mineralocorticoid activity

We then studied the in vivo capacity to recover

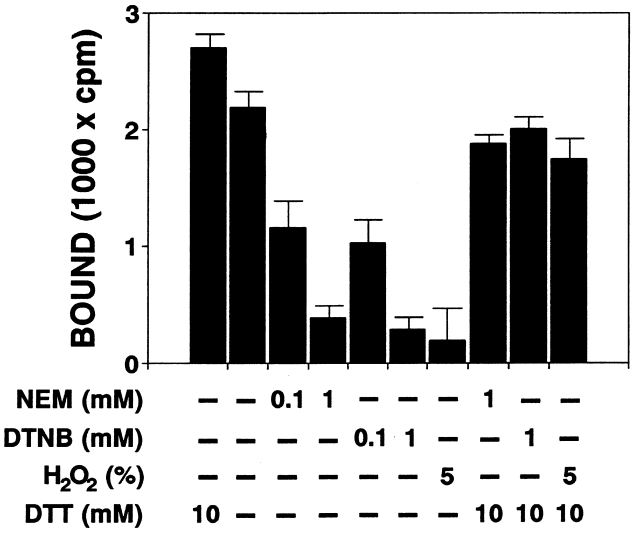

Fig. 5. Alkylation and oxidation inactivates mouse renal MR. Kidney cytosol was obtained from adrenalectomized mice in buffer lacking DTT. Alkylation of the MR (5 mg of cytosolic proteins) was carried out for 1 h at 8°C with either NEM or DTNB (0.1 mM and 1.0 mM) and receptor oxidation was induced in the presence of 5% v/v H_2O_2 for 30 min at 8°C. A steroid binding assay was then performed. Where indicated, alkylated or oxidized MR was reincubated in buffer supplemented with 10 mM DTT for 30 min at 0°C.

from the GSH depletion by co-treatment of L-(*S*,*R*)-BSO with GSH or GSH monoethyl ester. The mineralocorticoid biological effect was measured in mice injected with 2 μg ALDO/100 g as described for Fig. 1. Kidneys were excised quickly and the levels of GSH were measured in cytosol. Due to the presence of injected ALDO, the hormone binding cannot be measured in the same animals, so a second group of mice were treated in parallel (ALDO was not injected) to evaluate the steroid binding capacity in kidney cytosol. Results are shown in Table 3.

No significant differences were obtained for mineralocorticoid action, steroid binding, and tissue GSH levels in mice treated with the inactive diastereoisomer L-(*R*)-BSO or in mice treated with reduced GSH only. As expected, treatment with L-(*S*,*R*)-BSO not only depleted GSH tissue levels, but also inhibited both the ALDO-dependent mineralocorticoid effect and the steroid ALDO capacity in renal cytosol. Co-treatments with reduced GSH were ineffective to reverse these deleterious effects. In contrast, co-treatment with GSH monoethyl ester fully prevented the toxic effects of L-(*S*,*R*)-BSO by preserving the intra-

Table 3
Reversion of the inhibitory effect of L-(*S*,*R*)-BSO on the mineralocorticoid function by GSH monoethyl ester

Treatment	Na^+/K^+ ratio	[^{3}H]ALDO bound (fmol/mg of protein)	GSH (μmol/g of tissue)
Vehicle	0.18 ± 0.03	45 ± 2	2.3 ± 0.3
L-(*R*)-BSO (5 days)	0.23 ± 0.04	40 ± 4	2.0 ± 0.2
L-(*S*,*R*)-BSO (5 days)	0.55 ± 0.04	20 ± 4	0.3 ± 0.1
L-(*S*,*R*)-BSO (5 h)	0.59 ± 0.12	25 ± 9	0.2 ± 0.2
GSH (5 days)	0.19 ± 0.05	49 ± 6	2.5 ± 0.3
GSH ester (5 days)	0.17 ± 0.02	47 ± 5	4.0 ± 0.4
L-(*S*,*R*)-BSO+GSH (5 days)	0.42 ± 0.06	28 ± 5	0.4 ± 0.2
L-(*S*,*R*)-BSO+GSH ester (5 days)	0.18 ± 0.04	42 ± 3	3.0 ± 0.3
L-(*S*,*R*)-BSO+GSH ester (5 days)	0.20 ± 0.02	46 ± 7	4.2 ± 0.7
L-(*S*,*R*)-BSO+GSH ester (5 h)	0.21 ± 0.05	45 ± 5	2.7 ± 0.4
DEM (2 h)	0.60 ± 0.02	18 ± 2	0.2 ± 0.1
DEM+GSH ester (2 h)	0.22 ± 0.05	40 ± 4	4.1 ± 0.6

Adrenalectomized mice were injected (i.m.) for 5 days at intervals of 12 h (8.00 and 20.00 h) with L-(*S*,*R*)-BSO or L-(*R*)-BSO (5 mmol/kg/day). Controls were injected with vehicle. GSH or GSH monoethyl ester (6 mmol/kg/day) was also co-administered to some animals (9.00, 15.00 and 21.00 h). One group of mice was injected with a single dose of L-(*S*,*R*)-BSO and/or GSH ester and killed 5 h later. Acute treatments with DEM (3 mmol/kg) were conducted as explained in the text. The in vivo mineralocorticoid effect of 2 μg/100 g of ALDO was measured as urinary Na^+/K^+ ratio. Kidneys were then excised, homogenized, and GSH levels and [^{3}H]ALDO binding capacity were measured. The Na^+/K^+ ratio ranged between 1.69 ± 0.24 for all adrenalectomized mice injected with vehicle regardless of the treatment considered. Results represent means ± S.E.M. of five animals per group.

cellular levels of GSH. As can be seen in controls treated with GSH ester only or with L-(*R*)-BSO and GSH ester, the mineralocorticoid function did not undergo modifications with respect to controls.

In order to confirm that the effects observed on mineralocorticoid function were due to GSH depletion rather than unknown side effects of the drug, we depleted tissue levels of GSH by another method. Thus, mice were injected with DEM, a compound able to rapidly form GSH conjugates which are transported out of cells without inhibiting the biosynthetic pathway [17,31]. Preliminary experiments (not shown) demonstrated that the effect of a single i.p. injection of DEM (3 mmol/kg) resulted in a rapid depletion of renal GSH after 1 h (from 2.1 to 0.2 μmol/g). This depletion was rapidly reversed by re-

Table 4
Determination of reactive sulfhydryls in the MR immunoadsorbed from mouse renal cytosol after in vivo treatment with L-(*S*,*R*)-BSO

Treatment	Thiols (μmol/mg of protein)	Percentage of the control
Vehicle	0.92 ± 0.06	100
L-(*R*)-BSO	0.85 ± 0.10	92
L-(*S*,*R*)-BSO (5 h)	0.44 ± 0.12**	48
L-(*S*,*R*)-BSO+GSH ester (5 h)	0.97 ± 0.10	105
L-(*S*,*R*)-BSO (5 days)	0.38 ± 0.05*	41
L-(*S*,*R*)-BSO+GSH ester (5 days)	1.03 ± 0.15	112
L-(*S*,*R*)-BSO (5 h)+DTT in vitro	1.08 ± 0.18	117
L-(*S*,*R*)-BSO (5 days)+DTT in vitro	0.77 ± 0.03***	84

Adrenalectomized mice were treated for 5 days with 5.0 mmol/kg/day of L-(*S*,*R*)-BSO and/or 6 mmol/kg/day of GSH monoethyl ester. Kidneys were then excised, homogenized, and the MR was immunoadsorbed from cytosols with pAbhMR antibody as described in Section 2. The reactive sulfhydryl groups present in each stripped immunoadsorbed MR were quantified by duplicate with Ellman's reagent. In acute treatments, mouse kidneys were excised 5 h after the injection of a single dose of L-(*S*,*R*)-BSO and/or GSH monoethyl ester. The reactivation of oxidized thiols was achieved by incubation of the immunoprecipitated MR with 2 mM DTT (30 min at 0°C) followed by three washes with buffer. Immunoadsorptions performed with non-immune rabbit serum were used as blanks and subtracted from the respective $A_{412\,nm}$ obtained with pAbhMR. A molar absorptivity of 13 600 M^{-1} cm^{-1} was used to quantify reactive thiol groups. Results represent means ± S.E.M. for five mice per group. Different from control at: *$P < 0.001$; **$P < 0.010$; ***$P < 0.015$.

synthesis of GSH and 2.5 h after the administration of DEM the tissue levels of GSH were similar to those measured in the control. In the experiment shown in Table 3, mice were pretreated with 3 mmol/kg of DEM (i.p.), this dose being repeated after 1 h and 2 h. In parallel, another group of animals were co-treated with GSH ester (2.5 mmol/kg co-administered with DEM).

Mice treated with DEM exhibited low levels of GSH in kidney as well as an inhibited mineralocorticoid response to ALDO. The steroid binding capacity was also abolished. All these effects were prevented by co-treatment with GSH monoethyl ester, demonstrating that the depletion of GSH was in effect responsible for abrogating the mineralocorticoid effect.

When tissue levels of GSH, ALDO binding capacity, and Na^+/K^+ ratio are compared, significant differences ($P < 0.001$) are evidenced between the control group and mice treated with L-(*S*,*R*)-BSO (either co-treated or not with GSH) or DEM.

3.9. *Oxidation of renal mineralocorticoid receptor in L-(S,R)-BSO-treated mice*

In order to provide direct molecular evidence for receptor oxidation after GSH depletion, renal MR was immunopurified from cytosol obtained from L-(*S*,*R*)-BSO-treated mice and sulfhydryl groups were quantified with Ellman's reagent. To prevent the interference of co-immunoprecipitated proteins which belong to the steroid receptor heterocomplex (i.e. hsp90, p23, immunophilins, etc.) we stripped the immunoprecipitated receptor by incubation with high ionic strength. Immunoprecipitations performed with non-immune rabbit serum were used as blanks ($A_{412\,nm} = 0.02$) and subtracted from the $A_{412\,nm}$ obtained for each immunopellet. Results shown in Table 4 demonstrate that MR isolated from 5 h or 5 day treated mice yielded ~40–50% of reactive sulfhydryls as compared to untreated control. Consistent with the results presented in Table 3, co-treatment with GSH monoethyl ester fully preserved the thiol reactivity. In vitro reduction of the MR in the presence of DTT reversed the number of reactive sulfhydryls to control levels in acutely treated mice. In contrast, only a partial reversion (84% of the control, $P < 0.015$) was obtained with reincubated MR isolated from long-term treated animals. This observation is consistent with that analyzed in Fig. 4. No differences were obtained in the thiol reactivity when Ellman's reaction was performed in the absence of detergent (data not shown), evidencing that total and available protein sulfhydryls are indistinguishable.

To provide direct evidence that the inhibition of the mineralocorticoid effect may be due to the inability of oxidized MR to bind steroid, we used [^{3}H]DEX-mesylate as ligand. This synthetic steroid plays a double role of ligand and sulfhydryl reagent able to bind covalently to the steroid binding pocket of the receptor. Despite the fact that DEX-mesylate exhibits a 300-fold higher affinity for the GR ($K_d = 0.2$ nM) than for the MR ($K_d = 62$ nM), under conditions where the binding to the GR is blocked (i.e. in the presence of 100-fold excess of RU486) a significant steroid binding to the MR can be expected for a tracer concentration equal to 300 nM (83% of maximum binding for MR). Inasmuch as the GR is cleared from cytosol by specific immunoadsorption with BuGR2 antibody and the MR is then immunopurified with pAbhMR antibody, it can be predicted that the radioactivity associated with the pAbhMR immunopellet is due to steroid binding to MR. In effect, we have previously demonstrated [11] that there is no detectable amount of GR in kidney cytosol after three cycles of immunoadsorption with BuGR2. Accordingly, the levels of GR associated with [^{3}H]DEX-mesylate which were recovered after this treatment accounted for less than 5% of total radioactivity bound to the MR (data not shown). In addition, preliminary experiments performed in this work evidenced that the GR immunoadsorbed with BuGR2 was not labelled with [^{3}H]DEX-mesylate when kidney cytosol was incubated with [^{3}H] tracer in the presence of 30 μM RU486, demonstrating that under this experimental condition there is a full blockade of the GR. Therefore, the radioactivity associated with the pAbhMR immunopellet is certainly due to the [^{3}H]DEX-mesylate/MR complex.

Fig. 6A depicts autoradiograms of immunopurified MR labelled with [^{3}H]DEX-mesylate. Lane 1 shows no radioactivity in the pellet obtained with non-immune serum. Lane 2 shows a radioactive band in the immunopellet recovered from cytosol labelled with [^{3}H]DEX-mesylate in the absence of RU486. This band was not observed when 170-fold excess of ra-

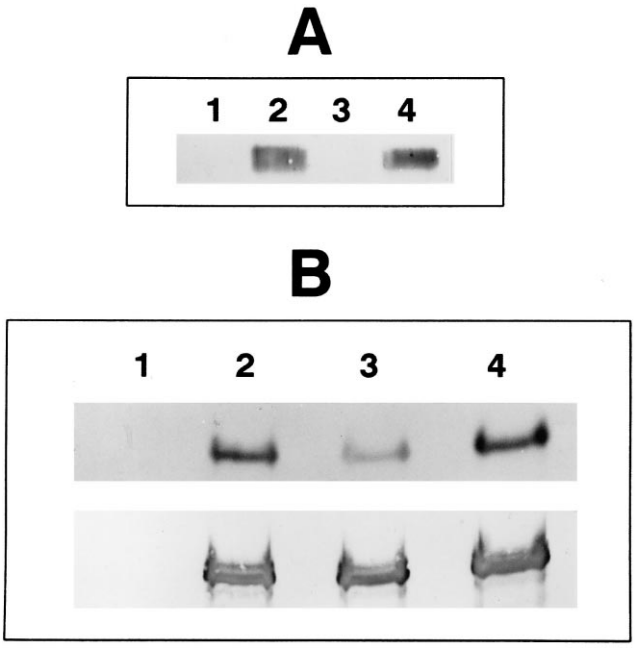

Fig. 6. Covalent binding of sulfhydryl-reactive ligand to renal mouse MR. (A) Immunoadsorption of mouse MR. Renal cytosol was labelled with 300 nM [^{3}H]DEX-mesylate for 3 h at 0°C as described in detail in Section 2. After adsorption of free ligand with charcoal/dextran, cytosols were precleared of GR with BuGR2 antibody and the MR was then immunoprecipitated with pAbhMR antibody (lanes 2–4). The immunopellets were analyzed by Western blot with 0.1% pAbhMR. Lane 1: immunoadsorption performed with non-immune rabbit serum. Lane 2: immunopellet from labelled cytosol. Lane 3: immunopellet from cytosol labelled in the presence of 50 μM radioinert ALDO. Lane 4: immunopellet from cytosol labelled in the presence of 30 μM radioinert RU486. (B) Immunopurification of labelled MR obtained from L-(*S*,*R*)-BSO-treated mice. Mice were treated with 5 mmol/kg/day of L-(*S*,*R*)-BSO for 5 days. Kidney cytosol was labelled with 300 nM [^{3}H]DEX-mesylate for 3 h at 4°C in the presence of 30 μM RU486. Labelled MR was specifically immunoprecipitated as explained for panel A, Western blotted (lower lane), and autoradiographed (upper lane). Conditions are: Lane 1: immunoadsorption performed with rabbit non-immune serum. Lane 2: MR from untreated mice. Lane 3: MR from L-(*S*,*R*)-BSO-treated mice. Lane 4: MR from mice cotreated with L-(*S*,*R*)-BSO and GSH monoethyl ester.

dioinert ALDO was added to the incubation medium (lane 3), demonstrating that the tracer is specifically bound to the steroid binding site of the MR. Lane 4 shows a radioactive band in the immunopellet obtained from cytosol labelled in the presence of 100-fold excess of RU486. Since densitometric scanning revealed that this band is not different from that observed in lane 2, we conclude that the autoradiogram shows the radioactive bands of [^{3}H]DEX-mesylate specifically bound to the MR. Nevertheless, we decided to conserve RU486 in the labelling medium for the next experiment to prevent any non-specific signal due to putative contamination with renal GR.

As previously shown in Fig. 4, reincubation of cytosol with DTT failed to recover all of the steroid binding capacity in renal cytosol obtained from mice treated with L-(*S*,*R*)-BSO for 5 days. Furthermore, Scatchard analysis performed with these preparations demonstrated that the total number of binding sites was decreased. This observations may be due to: (a) a certain percentage of oxidized MR present in the cells may be irreversibly inactivated, or (b) the concentration of receptor protein is decreased due to a generalized failure in protein synthesis and/or increased degradation. The experiment shown in Fig. 6B suggests that the former hypothesis is most likely. The upper bands correspond to an autoradiogram of immunopurified MR labelled with [^{3}H]DEX-mesylate, whereas the lower bands show the Western blot developed with horseradish peroxidase. Lane 1 is an adsorption performed with a non-immune serum, lanes 2–4 are immunoadsorptions performed with cytosols obtained from mice treated for 5 days with vehicle (lane 2), L-(*S*,*R*)-BSO (lane 3), and L-(*S*,*R*)-BSO plus GSH monoethyl ester. Densitometric scanning revealed that the relative densities of radioactivity are 1.00/0.43/1.22 for lanes 2, 3, and 4. The density ratios normalized as autoradiography signal/Western blot signal are respectively 1.00/0.44/0.96. These results are indicative that the concentration of receptor protein present in the kidney of L-(*S*,*R*)-BSO-treated mice does not decrease after 5 days of treatment, and also confirm the postulate that the binding of the ligand is abrogated because the MR is oxidized.

3.10. Depletion of GSH does not significantly affect the activity of proteins involved in the mineralocorticoid biological response

As noted in Section 1, the mineralocorticoid action involves proteins related to ion transport, energy metabolism, and glucocorticoid inactivating enzymes. In order to assess the effect of GSH depletion on the normal function of these proteins, we measured the

Table 5
Effect of L-(*S*,*R*)-BSO on kidney distal tubule

Parameter	Control	+L-(*S*,*R*)-BSO	
		5 h	5 days
Citrate synthase (μmol/mg per min)	2.42 ± 0.21	2.72 ± 0.44	2.33 ± 0.31
11β-Hydroxysteroid dehydrogenase (μmol/mg per min)	4.66 ± 0.53	4.44 ± 0.60	4.27 ± 0.92
Total ATPase (μmol/mg per min)	1.93 ± 0.14	1.81 ± 0.15	1.72 ± 0.22
Na,K-ATPase (μmol/mg per min)	0.47 ± 0.06	0.39 ± 0.06	0.48 ± 0.03
[^{3}H]Ouabain binding (cpm/mg)	488 ± 31	440 ± 41	646 ± 45*
[^{3}H]Amiloride binding (cpm/mg)	377 ± 26	360 ± 50	366 ± 42

Adrenalectomized mice were treated for 5 h or 5 days with 5 mg/kg of L-(*S*,*R*)-BSO, kidney distal tubules were then isolated, and some proteins were analyzed. Animals from the control group were injected with vehicle only. Results were normalized by mg of protein and shown as means ± S.E.M. of five mice per group. *Significantly different from control at $P < 0.010$.

activity of ATPases, citrate synthase, and 11β-hydroxysteroid dehydrogenase in kidney distal tubules isolated from normal and L-(*S*,*R*)-BSO-treated mice. We also measured the number of NaK pumps by binding of [^{3}H]ouabain, and the number of Na^+/H^+ antiport by binding of [^{14}C]amiloride. Results are shown in Table 5. As can be seen, only the number of total NaK pumps was significantly affected in kidney obtained from long-term treated mice. Even when the Na,K-ATPase activity measured in this group was similar to the untreated control group, the [^{3}H]ouabain binding was 30% higher, suggesting that the normal Na,K-ATPase activity was preserved by increasing the total number of pumps.

4. Discussion

Our results strongly support the notion that the in vivo oxidation of the renal MR leads to an abrogated mineralocorticoid biological response as a consequence of the inability of the receptor to bind agonist. Extensive studies have revealed that oxidized proteins are recognized by proteases and completely degraded (see [32] for a review); however, this may not be the case for the oxidative MR inactivation observed for long-term treatment with L-(*S*,*R*)-BSO. We have previously demonstrated [12] that, even when oxidized rat MR is unable to bind steroid, it is much more stable to thermal degradation in a cell-free system than the 'reduced' form of MR. According to those experiments, sulfhydryl reagents abolished the steroid binding ability of MR in a completely reversible manner upon the addition of reducing agents after incubations at 25°C, a temperature which allows extensive proteolysis in a cell-free system. In the present report, we provide direct evidence that the receptor protein remains stable in a non-reducing environment, although it is unable to bind ligand.

In all the cases studied here, the harmful effects on the mineralocorticoid response generated by GSH depletion were fully prevented by co-treatment with GSH ester, reinforcing the concept that oxidative stress inhibits the MR-dependent biological response.

In vitro studies performed previously on native rat MR and recombinant hMR demonstrated that a low redox potential greatly affects the steroid binding capacity [12,14,15]. The essential role of cysteines in MR for ligand binding was demonstrated in native rat receptor by conventional biochemical approaches [12,13] and in overexpressed recombinant receptor by site-directed mutagenesis studies [14]. Therefore, a deleterious effect upon oxidative stress was inferred from those studies. In this work, we show that these structural studies correlate with the in vivo biological response, a situation where the MR is not only regulated in its own physiological milieu, but more importantly where compensatory mechanisms may be triggered as a direct response to the oxidative stress. One example of these compensatory mechanisms may be found in the fact that the total activity of Na,K-ATPase is not altered after 5 days of GSH depletion. However, this is achieved by increasing the number of pumps, as evidenced in Table 5. Therefore, a proportional decrease in the specific activity of these pumps may be inferred.

It has been reported [33] that the full-length im-

munoreactive estrogen receptor can be extracted from primary human breast cancer tumors. However, this intact receptor is unable to bind to its cognate estrogen response element. Interestingly, this effect was reversed by incubation with an excess of DTT. These results not only demonstrate that the oxidized protein is not cleaved, but also suggest that the transactivational activity of the estrogen receptor may be modulated by changes in the intracellular redox potential.

The role of a reducing milieu on steroid receptors has also been demonstrated for the GR in experiments performed in vitro. It is known that the extraction of rat liver cytosol with charcoal inactivates the glucocorticoid binding capacity and receptors can be reactivated to the steroid binding state by an endogenous NADPH reducing system and an endogenous heat-stable activating factor identified as thioredoxin (reviewed in [34]).

Recently, we demonstrated that *S*-nitrosylation of the GR by nitric oxide decreases the steroid binding capacity [35]. This inhibition does not occur if the GR is previously bound to the ligand or when the redox potential of the medium is lowered. We concluded that the presence of steroid in the hormone binding pocket prevented such *S*-nitrosylation, and this led us to speculate about why glucocorticoids fail to exert their effects in septic shock, a situation where significant amounts of nitric oxide are generated. Consistent with this idea, glucocorticoids are beneficial in the treatment of this pathological condition if they are administered previous to the onset of septic shock.

It is known that the DNA binding domain of many transcription factors (steroid receptors, AP-1, p53, c-myb, NF-κB, Sp-1, E2, etc.) are also redox-sensitive, mainly via oxidation/reduction of critical cysteine residues within their zinc-finger loops [36,37]. There are also essential cysteines in the hormone binding domain of the steroid receptors. However, the sensitivity to oxidation of those amino acids in, i.e., the estrogen receptor [33] or the GR [35,38], is not as evident as in the MR. Thus, the hormone binding is abolished upon receptor alkylation or treatment with H_2O_2 in all the steroid receptors, but under more drastic conditions than those required for the MR. This suggests that thiol groups involved in the steroid binding domain may be more protrusive in the MR than in the other steroid receptors. This hypothesis is also supported by the experiments shown here in Table 2.

It is recognized that GSH can protect cells against oxidation, heavy metal ions, radiation, and certain toxic compounds [16]. Such protection is abolished by GSH depletion. In this sense, L-(*S*,*R*)-BSO seems to be a very useful and specific tool to study the GSH action. It has been reported that treatments with L-(*S*,*R*)-BSO were also effective to deplete GSH in newborn rats, adult mice, guinea pigs, and human cells [17,39–41]. Consistent with those studies, we show here that co-treatment with GSH ester was effective in increasing tissue GSH levels and, more importantly, in recovering the mineralocorticoid response in mice treated with L-(*S*,*R*)-BSO. On the other hand, treatment with GSH was ineffective. This may be ascribed to the fact that when the glycine carboxyl group of GSH is esterified the molecule exhibits better properties for GSH delivery and a slower rate of extracellular breakdown [16].

Our results provide clear evidence that the oxidative stress generated by tissue depletion of glutathione abrogates the in vivo biological response triggered by ALDO, mainly due to the fact that oxidized renal MR is unable to bind steroid. Inasmuch as glutathione depletion is systemic (Table 1), it is entirely possible that a similar mechanism of inhibition for the MR-dependent biological response may take place in other target organs which also express the MR, such as those studied in Table 2. One question which still remains unanswered is whether the reversible inactivation of the MR by oxidation is simply the obvious consequence of the amino acid modification under certain abnormal circumstances or is part of a more sophisticated mechanism to regulate receptor availability.

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