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Impairment of Mineralocorticoid Receptor (MR)-dependent Biological Response by Oxidative Stress and Aging

CORRELATION WITH POST-TRANSLATIONAL MODIFICATION OF MR AND DECREASED ADP-RIBOSYLATABLE LEVEL OF ELONGATION FACTOR 2 IN KIDNEY CELLS*

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Acute and chronic treatments of mice with the glutathione-depleting agent, L-buthionine-(*SR*)-sulfoximine (BSO), impaired the mineralocorticoid receptor (MR)-dependent biological response by inhibiting aldosterone binding. This steroid-binding inhibition was fully reversed when reducing agents were added to kidney cytosol obtained from mice treated for 5 h, but it was only partially reversed in cytosol obtained from mice treated for 10 days. Although the oligomeric structure of the MR-hsp90 heterocomplex was always unaffected, a decreased amount of MR protein was evidenced after the long term treatment. Such a deleterious effect was correlated with a post-translational modification of MR, as demonstrated by an increased level of receptor carbonylation. In addition, a failure at the elongation/termination step was also observed during the receptor translation process in a reticulocyte lysate system. Thus, a high polyribosomes/monomers ratio and both increased proteolysis and decreased ADP-ribosylatable concentration of elongation factor 2 (EF-2) were shown. Importantly, similar observations were also performed *in vivo* after depletion of glutathione. Notwithstanding the EF-2 functional disruption, not all renal proteins were equally affected as the MR. Interestingly, both EF-2 and MR expressed in old mice were similarly affected as in L-buthionine-(*SR*)-sulfoximine-treated young mice. We therefore propose that a dramatic depletion of glutathione in kidney cells mimics the cumulative effect of aging which, at the end, may lead to a renal mineralocorticoid dysfunction.

The biological effects of aldosterone (ALDO)[1] are mediated by the mineralocorticoid receptor (MR), a ligand-dependent transcription factor that belongs to the steroid receptor class of nuclear receptors. The transcriptional activation of the MR in epithelial cells triggers a series of events that are responsible for the regulation of the internal medium, *i.e.* Na^+ and H_2O retention and K^+ and H^+ elimination.

Steroid receptors exist as nuclear or cytoplasmic heterocomplexes associated to the 90-kDa heat shock protein (hsp90) chaperone system (1, 2). Regardless of its subcellular localization, this association stabilizes the receptor in its hormone binding and transcriptionally inactive form. It is thought that upon ligand binding, the steroid receptors undergo a conformational change that leads to the dissociation of the hsp90-heterocomplex, dephosphorylation, dimerization, translocation into the nucleus (for cytoplasmic receptors), hyperphosphorylation, and binding to specific hormone-responsive elements. Nonetheless, the actual temporal sequence of this cascade of events remains unclear. In contradiction to what was previously thought, it has been shown that the dissociation of the hsp90-heterocomplex upon steroid binding is not necessarily the first step in the signaling pathway (3, 4). Consistent with this observation, it has also been postulated that the receptor-hsp90 complex requires intact cytoskeletal tracks to move efficiently toward the nucleus (5). For this translocation process, the activity of the Ser/Thr phosphatases seems to play a key role (3, 6, 7), and cytoplasmic dynein may be the motor protein required to move the complex on the filaments (8, 9).

Interestingly, the MR nuclear translocation is abrogated by oxidative stress in intact kidney cells (2). Several studies (10–13) performed *in vitro* with both cell-free systems and cells in culture have demonstrated that cysteine groups on the MR play an essential role in steroid binding. We have recently provided direct evidence (14) that the MR function is also affected *in vivo* in a similar manner as shown *in vitro*. Thus, inhibition of ALDO binding to renal MR was observed after administering mice a transition state inhibitor of γ-glutamylcysteine synthetase, L-buthionine-(*SR*)-sulfoximine (BSO). The consequent GSH deficiency paralleled the inhibition of the mineralocorticoid biological responses to the same extent after both short (5 h) and long (5 days) periods of treatment. We demonstrated that this effect was due to the inhibition of the steroid binding to MR due to oxidation of essential cysteine groups rather than changes on the receptor protein concentration. However, we subsequently observed that the level of expression of renal MR is systematically decreased after GSH depletion for longer times (*i.e.* 10 days). Inasmuch as a wide variety of renal disorders involve the overproduction of reactive oxygen species (15–17), a decreased level of MR expression may be relevant to understand the regulation of the critical acid

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[1] The abbreviations used are: ALDO, aldosterone; MR, mineralocorticoid receptor; hsp90, 90-kDa heat shock protein; BSO, L- buthionine-(*SR*)-sulfoximine; CH, cumene hydroperoxide; GSH, reduced glutathione; GME, glutathione monoethyl ester; MDA, malondialdehyde; EF-2, elongation factor 2; Pt, total peptides; Pn, nascent peptides; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; SGK, serum- and glucocorticoid-dependent kinase.

base and electrolyte balance under normal or pathological circumstances (*e.g.* oxidative stress, metal intoxications, aging, etc.).

A profound imbalance between oxidants and antioxidants has been related to renal disorders, from minimal change nephritic syndrome to obstructive nephropathy (16, 18). Moreover, patients with end-stage renal failure on maintenance hemodialysis are chronically exposed to the oxidative stress generated by reactive oxygen species due to perdialytic neutrophil activation (19). Increased concentration of malondialdehyde (MDA) (20), an intermediate product generated by lipid peroxidation, and decreased levels of GSH (15) were also found in plasma and erythrocytes of hemodialyzed patients.

Because the MR is not an exported protein, a decreased rate of protein synthesis and/or an increased protein degradation in renal cells may explain the observed diminution of MR levels under chronic oxidative conditions. The proposition that oxygen radical-mediated oxidation is a marking step in protein turnover is supported by several observations (reviewed in Refs. 21 and 22 and references therein) so that most of the studies were mainly focused on protein degradation. In agreement with this notion, it has been demonstrated recently that the turnover of oxidized proteins is enhanced in fibroblasts via proteosomal activity (23). On the other hand, the molecular mechanism for protein synthesis failure during the onset of oxidative stress has not been extensively studied and, consequently, is not completely defined. Because of this fact and also because oxidized MR seems to be more stable to thermal degradation (although incapable to bind steroid) than its "reduced" form (10), we decided to analyze the effect of oxidative stress generated by GSH depletion on the individual steps of polypeptide synthesis in kidney. By using the GSH-depleting agent BSO as an experimental model, we aimed to study the expression of renal MR, and we correlated this process with the elongation phase of protein synthesis under *in vivo* and *in vitro* conditions. Because the elongation phase only requires a small number of factors as compared with the initiation step, we also focused our analysis on the MR translation process. In particular, we searched for putative post-translational modifications of elongation factor 2 (EF-2), the main protein implicated in the elongation step. Finally, we discuss our molecular findings from the perspective of an integrated process that may take place under normal and pathological conditions.

MATERIALS AND METHODS

Reagents—[1,2-^{3}H]ALDO (50 Ci/mmol), [U-*adenosine*-^{14}C]NAD (850 mCi/mmol), NaB[^{3}H]$_4$ (63 Ci/mmol), [^{35}S]methionine (1,000 Ci/mmol), L-[3,4-^{3}H]valine (55 Ci/mmol), and ^{125}I-conjugated counterantibodies were from PerkinElmer Life Sciences. Reduced GSH, GSH monoethyl ester (GME), L-buthionine-(*SR*)-sulfoximine (BSO), cumene hydroperoxide (CH), protein A-Sepharose, butylated hydroxyanisole, α-tocopherol, deferoxamine, mannitol, cysteine, sodium ascorbate, catalase from bovine liver, and diphtheria toxin from *Corynebacterium diphtheriae* were from Sigma. RU486 was a kind gift from Roussell-Uclaf (Romainville, France). Complete-Mini^{TM} protease inhibitor mixture was from Roche Molecular Biochemicals. DNA encoding for full-length human MR was generously provided by Dr. R. Evans (24). TNT Quick-coupled Transcription-Translation kit was from Promega Corp. (Madison, WI). Rabbit polyclonal antibodies against EF-2 (25) and the MR (26) were described previously. The mouse monoclonal IgG antibody against hsp90 was purchased from StressGen (Victoria, British Columbia, Canada). Donkey anti-rabbit and goat anti-mouse IgG-horseradish peroxidase antibodies were from Pierce. Chemiluminescence reagents were purchased from Amersham Biosciences.

Depletion of GSH—BSO was dissolved in ethanol/propylene glycol/saline solution (3:5:32). Two daily intramuscular injections of 2.5 mmol BSO/kg were given to BALB/c mice (see below) at intervals of 12 h (8:00 and 20:00 h) during the indicated times. In co-treatments with GME, three doses of 2.0 mmol/kg of the ester were also injected (8:00, 14:00, and 20:00 h). The ALDO-dependent sodium retention and potassium elimination were measured in urine samples collected as described previously (14, 27).

Steroid Binding Assays—Thirty-day-old and 18-month-old male BALB/c mice were adrenalectomized by dorsal approach and maintained with Purina Diet 1, saline solution supplemented with 50 μg/ml dexamethasone, and freshwater *ad libitum*. Two days before sacrifice, the steroid was omitted from the saline solution, and food was removed the previous night. Kidneys were excised after extensive perfusion with ice-cold phosphate saline solution, and renal cortex-medulla interphases were homogenized in 1 volume of buffer MOPS/Mo (20 mM MOPS, 5 mM EDTA, 2 mM EGTA, 10% glycerol, 2 mM DTT, 20 mM Na_2MO_4, at pH 7.5) containing 1 tablet of protease inhibitor mixture per 5 ml of solution. Homogenates were centrifuged at 67,000 $\times$ *g* for 45 min at 3 °C, and the supernatant of this centrifugation was referred to as cytosol. Renal cytosol was incubated for 12 h at 0 °C with 20 nM [^{3}H]ALDO and 1.0 μM RU486 to mask the slight cross-reaction of ALDO with the glucocorticoid receptor. A 500-fold excess of radioinert ALDO was used to determine the nonspecific binding (20% of the total). Bound steroid was separated from free steroid by adding 1 volume of 2% charcoal, 0.2% dextran 15–20. ALDO binding to kidney MR was measured in vivo as described before (14, 28). Briefly, 30-day-old male mice were treated with vehicle, BSO, or BSO and GME for the indicated periods. An intraperitoneal injection of 10 μCi of [^{3}H]ALDO and 20 μg of RU486 ($\pm$30 μg ALDO) was given. Kidneys were removed after 20 min and homogenized in buffer MOPS/Mo lacking DTT. Free [^{3}H]ALDO was cleared from cytosol by adsorption with charcoal/dextran, and the samples were divided into equal fractions. The specific binding was measured either without further treatment or after reincubating cytosol with 10 nM [^{3}H]ALDO ($\pm$500-fold ALDO) for 3 h at 0 °C in the absence or presence of 2 mM DTT. Free tracer was adsorbed with charcoal, and the radioactivity was measured.

Renal Polyribosomal Profiles—Polyribosomes and monomeric ribosomes were analyzed in post-mitochondrial supernatants treated with deoxycholate (~65% of the total renal RNA) by using a 20–40% linear sucrose gradient as described previously (25). The RNA profiles were monitored by measuring the absorbance at 260 nm and then semiquantified by planimetry.

Incorporation of [^{3}H]Valine into Total and Nascent Polypeptide Chains—The procedure for the determination of total proteins was similar to the one described previously (25) for liver. Mice were anesthetized with pentobarbital (50 mg/kg); the abdomen was opened, and 25 μCi of L-[3,4-^{3}H]valine in 200 μl of saline solution was injected in the renal artery. Tissue samples were taken at the indicated times and immediately frozen under liquid nitrogen until homogenization in 3 volumes of 0.3 M sucrose. Under these experimental conditions, the radioactivity was detected in kidney within 5–10 s after the injection of radioactive valine and continued at an approximately linear rate for 2.5–3.0 min. Therefore, we began to obtain tissue samples 20 s after the injection and during a total period of 2 min. Kidney homogenates in 0.3 M sucrose were diluted with 1 volume of a buffer containing 100 mM Tris and 2 mM magnesium acetate and centrifuged for 15 min at 4,000 $\times$ *g*. Two ml of the supernatant were mixed with 0.2 ml of 20% sodium deoxycholate, incubated on ice for 30 min, and then centrifuged at 8,000 $\times$ *g* for 15 min. Proteins in the resultant supernatant were used to quantify total and nascent chains. To measure radioactivity of total renal protein, an aliquot of this supernatant was treated with 10% trichloroacetic acid at 90 °C for 1.5 h. Precipitated proteins were redissolved with protosol, and the radioactivity was counted. The incorporation of labeled valine into nascent polypeptides was determined layering 1.5 ml of supernatant on 6 ml of a medium containing 1 M sucrose, 1 mM magnesium acetate, and 1 ml of a 100,000 $\times$ *g* supernatant obtained by 1 h of ultracentrifugation of kidney homogenate (1:2) in 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl and 0.25 M sucrose (pH 7.3). The mixture was centrifuged at 25,000 $\times$ *g*, and the resultant pellet was resuspended in 1 ml of distilled water. Proteins were precipitated and the radioactivity counted as described above.

Immunoprecipitation of MR—We followed a standard technique as described previously (3, 14). Briefly, renal cytosol was incubated with the rabbit anti-MR immune serum (or non-immune rabbit serum) prebound to protein A-Sepharose and washed four times with ice-cold MOPS buffer supplemented with 100 mM NaCl and 0.01% Nonidet P-40, and MR and hsp90 were identified by Western blot analysis.

In Vitro Transcription and Translation of the MR—The procedure was performed using the TNT Quick-coupled Transcription/Translation kit from Promega Corp. (Madison, WI) according to the manufacturer's instructions. Recombinant phMR3750 DNA encoding for full-length human MR (24) inserted into polylinker *Eco*RI site of pGEM4 (Promega Biotech) was used as a template for transcription with T7 polymerase

TABLE I

Oxidative stress abrogates the mineralocorticoid biological effect

Adrenalectomized 30-day-old or 18-month-old mice were treated with BSO or BSO and GME for the indicated times. Controls were treated with vehicle only. The mineralocorticoid effect (Na^+/K^+ ratio) was measured *in vivo* after injecting a saturating dose of ALDO as described under "Materials and Methods." Contents of GSH in renal cytosol and MDA and CO were also quantified in microsomal fractions. Results are the mean ± S.E. ($n = 6$).

Condition	Renal GSH[a]	MDA[b]	CO[b]	Na^+/K^+ ratio[c]
30-day-old mice	2.51 ± 0.21	16.1 ± 2.9	351.3 ± 33.1	0.17 ± 0.04
BSO				
5 h	0.34 ± 0.15[d]	37.5 ± 9.3[e]	544.4 ± 22.7[d]	0.44 ± 0.10[d]
3 days	0.18 ± 0.06[d]	40.1 ± 1.8[d]	635.9 ± 21.9[d]	0.50 ± 0.11[d]
10 days	0.15 ± 0.08[d]	61.4 ± 1.9[d]	729.6 ± 45.0[d]	0.58 ± 0.05[d]
BSO + GME				
5 h	2.75 ± 0.14	18.1 ± 3.0	370.7 ± 20.8	0.15 ± 0.02
3 days	4.04 ± 0.61[e]	14.4 ± 1.2	318.0 ± 13.1	0.19 ± 0.09
10 days	4.62 ± 1.01[e]	13.7 ± 3.1	304.1 ± 21.2	0.21 ± 0.12
18-Month-old mice	1.87 ± 0.45[e]	36.0 ± 4.2[d]	608.3 ± 32.1[d]	0.37 ± 0.03[d]

[a] Values are given in μmol/g of renal tissue.
[b] Values are given in mmol/mg of microsomal protein.
[c] The Na^+/K^+ ratio in control mice was 1.27 ± 0.14.
[d] Values different from control (30-day-old mice) at $p<0.001$.
[e] Values different from control at $p < 0.010$.

followed by translation in the presence of [^{35}S]methionine. Proteins were resolved by SDS-PAGE and autoradiographed. When the incubations were performed in the presence of oxidants, the reticulocyte lysate was preincubated for 60 min at 25 °C with either 2.0 mM CH and/or 5.0 mM BSO. In the protection assays against oxidants, a reducing solution was also simultaneously added to the medium at the following final concentrations: 20 μM butylated hydroxyanisole, 200 μM α-tocopherol, 5 mM GSH, 5 mM cysteine, 2 mM DTT, 1 mM sodium ascorbate, 20 mM mannitol, 3 mM deferoxamine, and 0.5 mg/ml catalase.

ADP-ribosylation of EF-2—ADP-ribosylation was performed by a modification of the method used by Galicka *et al.* (29). Renal EF-2 was immunoprecipitated from renal cytosol with the rabbit polyclonal antibody raised against EF-2 precoupled to protein A-Sepharose. The immune pellet was washed twice with MOPS buffer containing 100 mM NaCl and 0.01% Nonidet P-40 and twice with 20 mM Tris buffer at pH 7.4. The pellets were incubated for 1 h at 37 °C with 50 μl of a solution containing 20 mM Tris, 10 mM DTT, 10 μM [^{14}C]NAD, and 5 μg of diphtheria toxin. The pellets were washed four times with 1 ml of 20 mM Tris buffer, and the radioactivity incorporated to the immunopurified EF-2 was counted.

Purification of EF-2—EF-2 was isolated from the ribosome-free extract of rabbit reticulocyte lysate as described by Ryazanov and Davydova (30). The purity of the final preparation was verified by SDS-PAGE followed by Coomassie Blue G-250 staining and parallel Western blotting with anti-EF-2 antibody. The rate of incorporation of [^{35}S]Met to hMR in rabbit reticulocyte lysate system supplemented with pure EF-2 was measured after alkaline hydrolysis followed by trichloroacetic acid precipitation as described previously (31).

Miscellaneous—Renal GSH concentration was quantified by an enzymatic assay as described previously (14). Carbonyl content in either renal microsomes or immunopurified proteins was measured by reduction with tritiated sodium borohydride according to Lenz *et al.* (32). MDA was measured with the thiobarbituric standard method described by Estebauer and Cheeseman (33). Statistical tests were carried out by analysis of variance followed by Bonferroni analysis.

RESULTS

Effect of GSH Depletion on the Mineralocorticoid Response—Table I shows that the renal concentration of GSH was dramatically decreased when mice were treated with the γ-glutamylcysteine synthetase inhibitor, BSO. Such an efficient depletion was achieved as soon as 5 h after a single injection of BSO. On the other hand, a co-treatment with the cell-permeable ester GME fully prevented the deleterious effect of BSO by preserving (and even increasing) the intracellular levels of GSH. In agreement with the onset of an oxidative intracellular milieu, an increased concentration of both MDA, a product generated by the break down of hydroperoxides, and protein carbonyls were also measured after acute (5 h) and prolonged (3 and 10 days) treatment. On the other hand, the levels of these two markers of oxidation were indistinguishable from untreated controls when mice were co-treated with BSO and GME.

To evaluate the *in vivo* mineralocorticoid effect, a saturating dose (28) of 2 μg of ALDO per 100 g of body weight was injected, and the anti-natriuretic and kaliuretic effects were measured. ALDO decreased the Na^+/K^+ urinary ratio by 80% in control adrenalectomized mice. However, the biological response to ALDO was attenuated after 5 h of BSO injection, so a 2.5-fold higher Na^+/K^+ ratio was measured. This effect was more significant for longer periods of treatment (3 and 10 days). Taken together, these results clearly confirm (14) that the depletion of renal GSH impairs the mineralocorticoid biological response. Interestingly, the data shown in Table I also suggest a similar effect of aging on the mineralocorticoid response. Thus, 18-month-old animals also exhibit high levels of protein carbonyls and MDA, as well as a decreased biological response to ALDO as compared with 30-day-old animals.

In Vivo [^{3}H]ALDO Binding Assay—To evaluate the MR binding capacity *in vivo*, control and BSO-treated mice were injected with [^{3}H]ALDO, and kidneys were excised after 20 min, a time when renal radioactivity reaches a maximum level in kidney (28). Renal cytosol was obtained in MOPS/Mo buffer without DTT; free tracer was cleared by adsorption with charcoal/dextran, and the samples were divided into equal fractions. The specific binding (Fig. 1) was measured either without further treatment or after a reincubation of cytosols with [^{3}H]ALDO in the absence or presence of 2 mM DTT. As expected, the treatment with BSO decreased the steroid binding capacity of MR under *in vivo* conditions, whereas this inhibition was fully prevented by co-treatment with GME. When radiolabeled cytosols were reincubated *in vitro* with [^{3}H]ALDO, the specific binding remained unchanged (*gray bars*), indicating that the *in vivo* ALDO labeling had saturated the MR binding capacity. Consistent with the notion that an oxidative milieu inhibits the steroid binding capacity of MR by oxidation of essential cysteine groups, reincubation of these cytosols in the presence of DTT recovered the specific binding of cytosolic MR obtained from BSO-treated mice (*black bars*). Importantly, such recovering was total in mice treated for 5 h, but it was only partial in cytosols obtained from mice treated with BSO for 3 and 10 days. Thus, ~20 and 35% of the MR steroid binding capacity was not recovered, respectively. Again, old mice ex-

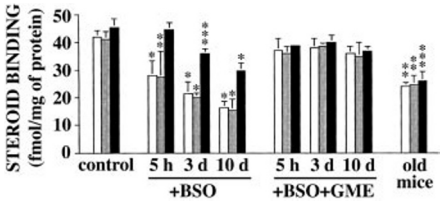

FIG. 1. ***In vivo* ALDO binding to kidney MR.** Thirty-day-old male mice were treated with vehicle (*control*), BSO, or BSO and GME for the times indicated *below* the graphs. Then an intraperitoneal injection of 10 μCi of [^{3}H]ALDO and 20 μg of RU486 ($\pm$30 μg ALDO) was given. Kidneys were removed after 20 min and homogenized in a buffer lacking DTT. Free [^{3}H]ALDO was cleared by adsorption with charcoal/dextran, and the samples were divided into equal fractions. The specific binding was measured either without further treatment (*white bars*) or after a reincubation of the cytosol for 3 h at 0 °C with 10 nM [^{3}H]ALDO ($\pm$500-fold ALDO) in the absence (*gray bars*) or presence (*black bars*) of 2 mM DTT. The ALDO binding capacity measured *in vivo* in 18-month-old mice is also shown (*old mice*). Results represent the means $\pm$ S.E. of four animals per group. Different from controls at *, $p < 0.001$; **, $p < 0.005$; and ***, $p < 0.010$.

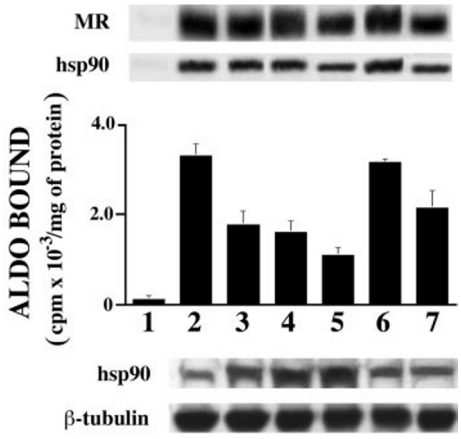

FIG. 2. **Co-immunoprecipitation of hsp90 with the MR.** Mice were treated with BSO alone or co-treated with BSO and GME for the indicated times. Kidney cytosol was then obtained, and the MR was immunoprecipitated. A Western blot for MR and co-immunoprecipitated hsp90 is shown at the *top* of the figure. An aliquot of this cytosol was used to perform a steroid binding assay (*bar graph*) and depicted as the means $\pm$ S.E. ($n = 4$). A second aliquot of cytosol was Western-blotted for hsp90 and β-tubulin (shown on the *bottom* of the graphic). Conditions are as follows. *1*, Non-immune pellet was obtained by incubation of control cytosol with a preimmune rabbit IgG antibody. *2*, Control cytosol was from untreated mice. Cytosol from BSO-treated mice for 5 h (*3*), 3 days (*4*), and 10 days (*5*). *6*, Cytosol was from mice co-treated with BSO and GME for 10 days. *7*, Cytosol was from 18-month-old mice. As compared with condition 2, differences of specific binding are significant at $p < 0.001$ for conditions 3–5, and $p < 0.005$ for condition 7.

hibited similar properties as those shown by long term BSO-treated young mice.

Association of MR with hsp90—Because the association of hsp90 with MR is an absolute requirement to bind steroid, we analyzed the co-immunopurification of hsp90 with renal MR after treatment with BSO. The *bar graph* in Fig. 2 depicts the ALDO binding capacity measured *in vitro* in cytosols obtained from control and treated mice, because these experimental results are similar to those shown in Fig. 1 under *in vivo* conditions. Moreover, a constant hsp90/MR optical density ratio (0.19 ± 0.02) was obtained for all the Western blots shown at the *top* of Fig. 2 after a densitometric scanning. These co-immunoprecipitation assays demonstrate that oxidative stress does not decrease the MR binding capacity by disrupting the association of MR with the chaperone complex. However, the amount of MR (and hence, hsp90) recovered in the heterocomplex after 10 days of treatment with BSO (condition 5) was one-third lower than the amount of MR protein recovered from either control cytosol (condition 2), cytosols from BSO-treated mice (conditions 3 and 4), or GME- and BSO-treated mice (condition 6). These results demonstrate that the decreased steroid binding capacity of MR measured in renal tissue was due to a lower concentration of MR rather than an oxidative disruption of the MR-hsp90 interaction. Western blot analysis of total cytosol resolved by SDS-PAGE (shown *below* the *bar graph*) evidenced that the cytosolic hsp90 concentration was greatly increased due to the onset of oxidative stress, and such induction was fully prevented by co-treatment with GME (condition 6). A Western blot for β-tubulin was also performed in the same samples, and no change was evidenced for this essential cytoskeletal protein.

Importantly, Fig. 2 also demonstrates that all the features observed in chronically stressed young mice were also present in untreated old mice (compare conditions 5 and 7), reinforcing the similarities pointed out before between oxidative stress and natural aging.

In Vitro Transcription and Translation of Human MR—The decreased concentration of MR obtained by chronic oxidative stress can be due to receptor degradation or a less efficient receptor synthesis. There are evidences in favor of both possibilities. Thus, it is known that radically mediated damaged proteins are often functionally inactive, and their unfolding was associated with enhanced susceptibility to proteases (22). However, we have reported previously (10) that the oxidized MR protein, although incapable to bind steroid, seems to be quite stable as compared with its reduced counterpart. Moreover, recent evidence supports the notion that protein synthesis may decline under oxidative conditions by changes in the polypeptide elongation rate (34, 35). Therefore, we focused our study on the various steps of protein synthesis under oxidative conditions.

We first analyzed the expression of a DNA template encoding for human MR by using the rabbit reticulocyte *in vitro* transcription/translation system. A main ^{35}S-labeled product can be seen as a 110-kDa band in Fig. 3*A*, as this molecular weight is compatible with the size of human MR (24). Importantly, this 110-kDa band was also revealed by Western blot with the anti-MR antibody (data not shown). Preincubation of reticulocyte lysate with BSO failed in affecting the MR translation in a significant form (Fig. 3*A*, compare *lane 2 versus 1*). In part, this failure may be due to the mechanism of action of the transition state enzymatic inhibitor. In effect, BSO is present in a post-mitochondrial medium that lacks an efficient source of peroxide radical products generated during, for example, an active oxidative metabolism. Then the simple inhibition of the GSH synthesis under these *in vitro* conditions may not be sufficient to affect significantly the translation machinery. That this may be the case is supported by the significant inhibition of MR expression achieved with cumene hydroperoxide (CH) (Fig. 3*A*, *lane 3*), a known generator of reactive oxygen intermediates (25, 36, 37), and also because of the potentiation obtained when both agents, BSO and CH, were used together (Fig. 3*A*, *lane 5*). As expected for a radically mediated effect, the CH-dependent inhibition of MR synthesis was abolished when the lysate was preincubated with CH in the presence of a reducing mixture (Fig. 3*A*, *lane 4*) containing 20 μM butylated hydroxyanisole, 200 μM α-tocopherol, 5 mM GSH, 5 mM cysteine, 2 mM DTT, 1 mM sodium ascorbate, 20 mM mannitol, 3 mM deferoxamine, and 0.5 mg/ml catalase. Inasmuch as the synthesis of MR was performed *in vitro,* two steps are then required to complete the process, template transcription followed by translation. It could be possible that a decreased production of MR may be due to a failure in the former process rather than in the translation step. Therefore, we per-

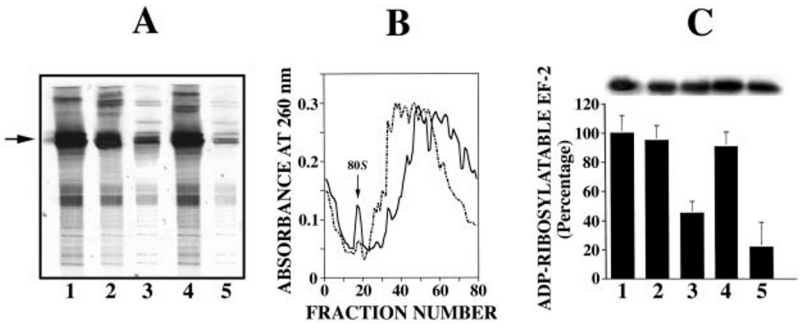

FIG. 3. ***In vitro* translation of human MR.** *A*, electrophoretic analysis of the translated product. The transcription/translation system was pretreated for 30 min at 25 °C as follows: *lane 1*, untreated control; *lane 2*, 5 mM BSO; *lane 3*, 2 mM CH; *lane 4*, 2 mM CH and the reducing mixture described under "Materials and Methods"; *lane 5*, 5 mM BSO and 2 mM CH. The translation reaction was performed in the presence of [^{35}S]methionine. Proteins were resolved by SDS-PAGEand autoradiographed. The *arrow* shows the band of MR when a Western blot revealed it. *B*, sucrose gradient of rabbit reticulocyte ribosomes. Polyribosomal profiles from normal lysate (*solid line*) and CH-treated lysate (*dotted line*) were determined as described under "Materials and Methods." The *arrow* shows the 80 S monomers. *C*, ADP-ribosylatable concentration of EF-2. EF-2 was immunoprecipitated from reticulocyte lysate pretreated in the same conditions as described for *A* (*lanes 1–5*). A Western blot of this immunoprecipitation is shown at the *top* of the *bar graph* for each condition. The active concentration of EF-2 was measured by using a standard reaction with [^{14}C]NAD and diphtheria toxin. The *bar graph* represents the means ± S.E. ($n = 3$) for the percentage of active EF-2 with respect to the untreated lysate (*condition 1*) standardized as 100% (11.3 ± 1.9 pmol/mg). *Conditions 3* and *5* are significantly different from *condition 1* at $p < 0.001$.

formed direct studies on the initiation and completion steps of the polypeptide synthesis by analyzing the polyribosomal profiles in reticulocyte lysates under normal and oxidative conditions. A representative linear sucrose gradient is depicted in Fig. 3*B*. It shows that an increased ribosomal state of aggregation was obtained under oxidative conditions. Thus, the polysomes/monomers ratio was 4-fold higher in treated lysates than in control samples (66 *versus* 16, respectively). The higher polysomes/monomers ratio observed in CH-treated lysate is the consequence of both a 2-fold increased amount of polysomes and also a 50% reduction of the single 80 S ribosome forms. It is known that the cycling between single ribosomal subunits (forms not involved in translation) and polyribosomes is very rapid (38, 39), and those ribosomes released at chain termination may either become monomeric ribosomes or may be converted into native subunits (31, 40) unless the initiation is suppressed, in which case accumulation of single ribosomes occurs (41–43). Therefore, the results shown in Fig. 3*B* agree with the notion that the elongation/termination step should be more affected by oxidative stress than the initiation step during the translation process.

The main protein implicated in the elongation step is the EF-2. Therefore, we analyzed the EF-2 level by Western blotting aliquots of reticulocyte lysate incubated with BSO and CH, and no differences were observed for the total concentration of EF-2 in any condition (data not shown). It is known that ADP-ribosylation of a peculiar diphthamide residue present on EF-2 abolishes its ability to translocate the peptidyl-tRNA from the A-site to the P-site on the ribosome (44). Furthermore, ADP-ribosylation of EF-2 with diphtheria toxin and NAD have been used as an indicator of the active EF-2 fraction (25, 29, 34, 45). Therefore, we immunopurified EF-2 from reticulocyte lysate and determined the amount of active protein. Fig. 3*C* demonstrates that the amount of ADP-ribosylatable EF-2 was reduced by 50 and 80% in CH- and CH/BSO-treated samples (conditions 3 and 5, respectively). This reduced level of active EF-2 was not observed when the incubation was performed in the simultaneous presence of the reducing mixture. Taken together, the results shown in Fig. 3 suggest that it is entirely possible that the decreased MR concentration observed in GSH-depleted mice may lie on the inability of renal cells to achieve efficiently the completion of the nascent protein, more specifically due to transformation of EF-2.

The Addition of Purified EF-2 Recovers the Transcription of hMR in Vitro—Given the number of proteins that are likely to be modified during oxidative stress, a direct role of EF-2 cannot be ensured from our previous experiments. Nonetheless, if the damage of EF-2 is one of the reasons for the observed decrease in hMR levels during the transcription, the addition of purified EF-2 to the transcription/translation system should correct such deficiency. Therefore, we first purified EF-2 from rabbit reticulocyte lysate and adjusted the amount to be added to the translation medium by comparison with the endogenous level of EF-2 present in reticulocyte lysate. The Western blot shown in Fig. 4*A* shows that 0.3 μl of purified EF-2 (referred to as 1×) matches the concentration of endogenous EF-2 in 5.0 μl of reticulocyte lysate, so that we used this relative amount of EF-2 as a reference. We then supplemented the CH-treated reticulocyte lysate employed as a transcription/translation system with purified EF-2. The hMR translation products are shown in Fig. 4*B*. As can be seen, the inhibitory effect of oxidative stress on the hMR translation was prevented when purified EF-2 was added to the incubation medium (Fig. 4*B*, compare *lane 3 versus lane 2*). On the other hand, the addition of purified EF-2 preincubated with 2 mM CH exhibited no effect on the level of expression of hMR (Fig. 4*B,* compare *lanes 4 versus lane 1*).

Fig. 4*C* depicts the rates of synthesis of hMR measured as incorporation of [^{35}S]methionine to the acid-insoluble fraction. The rate of synthesis of ^{35}S-labeled hMR was greatly decreased under oxidative conditions, whereas the addition of purified EF-2 to the medium was able to correct the translation process in a concentration-dependent manner. In contrast, when the translation mixture was supplemented with the same preparation of EF-2 that had been preinactivated with CH, the correction of the hMR translation was not observed. The purified EF-2 protein remains stable under the conditions used for the preincubation with CH (as judged by SDS-PAGE followed by Coomassie Blue staining), although its ADP-ribosylation by diphtheria toxin was fully abolished (data not shown).

Taken together, these results clearly indicate that EF-2 may be responsible for the observed decrease of hMR level during the translation process under oxidative conditions.

Renal Polyribosomal Profiles—In view of the previous observations, we next analyzed the polysome profiles in kidney cytosol after mice were treated with BSO. Results are shown in Fig. 5. Consistent with the above-described *in vitro* effect, Fig. 5*A* shows an increased ribosomal state of aggregation when oxidative stress was generated *in vivo* by treatment with BSO for 3 and 10 days (25 and 82% larger polyribosome peak,

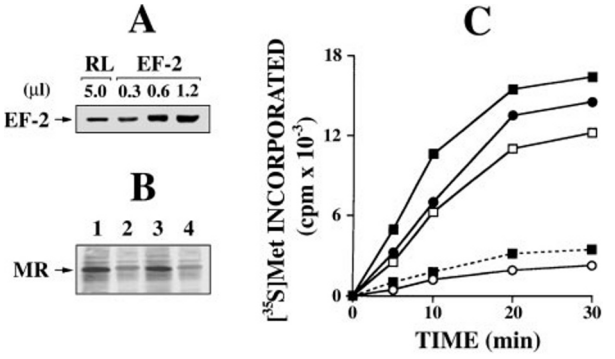

FIG. 4. **The addition of purified EF-2 to reticulocyte lysate prevents the harmful effect of a low redox potential medium on the translation process.** *A*, relative amount of purified EF-2. Various amounts (0.3, 0.6, and 1.2 μl) of the final preparation of purified EF-2 were compared by Western blotting with the endogenous level of EF-2 present in 5 μl of reticulocyte lysate. The ratio 0.3 μl of pure EF-2 to 5 μl of reticulocyte lysate is referred to as 1 time. *B*, translation of hMR in reticulocyte lysate system. The translation reaction was performed as described for Fig. 3*B* and autoradiographed for the [35S]Met-labeled hMR. Conditions are as follows: *1*, untreated control; *2*, lysate treated with 2 mM CH; *3*, lysate treated with 2 mM CH and supplemented with 2 times purified EF-2; *4*, lysate treated as in condition 3 but EF-2 was incubated for 1 h at 25 °C with 2 mM CH. *C*, rate of synthesis. The radioactivity associated to the trichloroacetic acid-insoluble product was measured as a function of the translation time. Conditions are as follows: untreated control (*solid circles*), lysate treated with 2 mM CH (*open circles*), lysate treated with CH and supplemented with EF-2 as follows: 1× (*open square*), 2× (*solid squares*, *solid line*), or 2× stored for 24 h in a buffer containing 2 mM CH (*solid squares*, *dotted line*). Results are the average of two independent experiments performed by duplicate.

respectively). On the other hand, the polyribosomal profile for BSO- and GME-co-treated mice was indistinguishable from that obtained with untreated mice (Fig. 5*B*). In addition, the polyribosomes/monomers ratio was also increased in 18-month-old mice (116%), strengthening the notion that aging is a process where cumulative damage by oxidation affects the protein synthesis machinery.

We then measured the polypeptide chain completion time. This parameter is accepted as a quantitative expression of the rate of peptide chain elongation and termination (25, 34, 46, 47). The radioactivity incorporated into both nascent peptides in polyribosomes (Pn) and total peptides (Pt) was plotted against the time after the injection of [3H]valine into the renal artery. The value of Pn for each animal was then divided by the corresponding value of Pt, and the Pn/Pt ratio was finally plotted against the time (Fig. 5*C*). If it is assumed that the radioactive amino acid will meet the ribosomes in the middle of the translation of a mRNA of average size (25, 46, 47), so the whole peptide on the ribosome should be labeled when a full cycle is completed. In turn, those chains that have been terminated and released will be only 50% labeled. After a second cycle, 1 full unit will increase the pool of released peptides, whereas the Pn/Pt ratio should have been reduced to 25%. Thus, the time required to reduce Pn/Pt from 50 to 25% should be representative of the average completion time (elongation and termination steps). This time is independent of both the number of ribosomes engaged in the process and variations in the initiation step and can be calculated directly from the slope of the function. In our hands, the average completion time increased from 58 to 98 s in mice treated with BSO for 10 days. Old mice evidenced a completion time equal to 158 s.

Damage of Renal MR and EF-2—We then measured the ADP-ribosylatable content of EF-2 immunopurified from kidney cytosol of BSO-treated mice. Fig. 6 shows that the active amount of EF-2 was reduced nearly 50% after 10 days of GSH depletion (*hatched bars*). In turn, the protein carbonyl content in the immune pellets was increased 2-fold (*black bars*). Both deleterious effects were totally prevented by GME. On the other hand, the content of carbonyls measured in MR immunopurified from BSO-treated mice (*white bars*) was increased 40% with respect to untreated animals. A similar level of MR carbonylation was also measured in 18-month-old mice.

In the experiment shown in Fig. 2, we demonstrated that the quaternary structure of the MR-hsp90 heterocomplex was not affected by oxidative stress. Those Western blots did not show lower molecular mass proteolytic fragments of MR. This observation agrees with the results shown in Fig. 3*A* and those described previously (10). On the other hand, Parrado *et al*. (34) have recently reported that rat liver EF-2 does undergo fragmentation upon the onset of oxidative stress induced by CH. As a consequence, we studied the putative fragmentation of cytosolic EF-2 in mouse kidney after treatment with BSO. Fig. 6*B* shows the protein profile obtained with the anti-EF-2 antibody in renal cytosol. Thirty-day-old untreated mice exhibited a main band of EF-2 at the expected molecular mass of 100-kDa (Fig. 6*B*, *lane 1*). Besides this band, cytosol from BSO-treated mice also exhibited a major proteolytic fragment at 39 kDa and two minor bands at 51 and 67 kDa (*lanes 2* and *3*). The full prevention of the EF-2 fragmentation observed in cytosol of GME co-treated mice (*lane 4*) proves that proteolysis of EF-2 *in vivo* depends on the onset of oxidative stress. Thus, old mice also exhibit a fragmentation pattern of EF-2 similar to that generated by the depletion of GSH in young mice (*lane 5*). However, the total amount of full-length EF-2 was also significantly decreased in old mice, an observation that is consistent with the significantly slower half-transit time of nascent polypeptides observed in Fig. 5*C* for this group of animals. Diminished EF-2 levels were also found in liver of CH-treated rats (34). Curiously, despite the lower full-length EF-2 level observed for old mice, Fig. 6*B* also shows that the amount of proteolytic fragments remained unchanged. This observation may be related to the inhibition of the proteosome activity described in certain old cells (23), which in turn leads to the cyclic accumulation of damaged and aggregated proteins.

DISCUSSION

In this work we demonstrated that oxidative stress impairs the mineralocorticoid biological response by two different molecular mechanisms. As supported by the experiments described in Table I and Figs. 1 and 2, one of these inhibitory mechanisms involves the post-translational modification of the receptor protein by oxidation. Consistent with the results reported in the literature (10–14), it is most likely that oxidization of essential cysteine groups is the main responsibility for such inhibition. In the short time (hours), the inhibitory effect observed by depletion of GSH can be fully reversed *in vitro* by incubating renal cytosol with DTT or can be totally prevented *in vivo* by co-administering GME. A second harmful mechanism able to affect the mineralocorticoid response was evidenced after several days of GSH depletion, and it affected the protein translation system at the elongation/termination steps.

Because polyribosomes can be formed *in vitro* (Fig. 3*B*) and *in vivo* (Fig. 5, *A* and *B*), and there was a decreased number rather than accumulation of monomers, it is unlikely that the initiation step can be as strongly affected as the completion step (41–43). In agreement with the notion that the elongation/termination step is affected by the onset of oxidative stress, the rate of translation *in vitro* was restored after addition of purified EF-2 to the incubation medium (Fig. 4). Moreover, the half-transit rate of nascent polypeptide chains in renal cells is almost twice as slow in BSO-treated mice as in the untreated controls. The observed effects on protein synthesis during the BSO-dependent depletion of renal GSH are similar to those

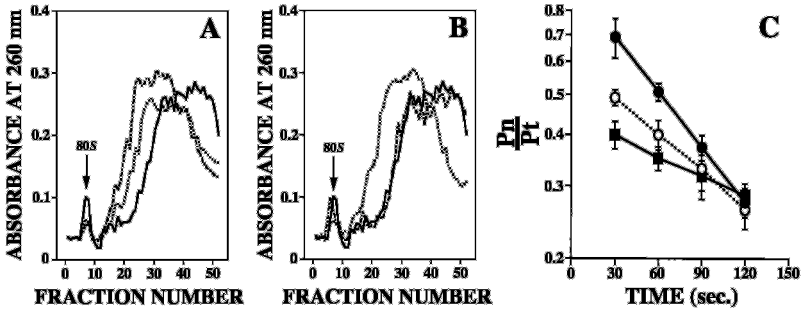

FIG. 5. **Polyribosomal profiles and polypeptide completion time in kidney.** A sucrose gradient of renal polysomes was performed after 30-day-old mice were treated under the following conditions (polysomes/monomers ratios are given in parentheses). *A*, untreated controls (*solid line*) (13); mice treated with BSO for 3 days (*dashed line*) (40), or 10 days (*dotted line*) (59). *B*, untreated 30-day-old controls (*solid line*) (13), untreated 18-month-old mice (*dashed line*) (47); 30-day-old mice co-treated with GME and BSO for 10 days (*dotted line*) (12). *C*, [^{3}H]valine incorporated into renal Pn and Pt was measured in 30-day-old mice treated for 10 days with either vehicle (*black circles*) or BSO (*white circles*). The Pn/Pt ratio for untreated 18-month-old mice is also shown (*black squares*). The completion time was calculated from the slope of each function. The points represent the means ± S.E. of four mice.

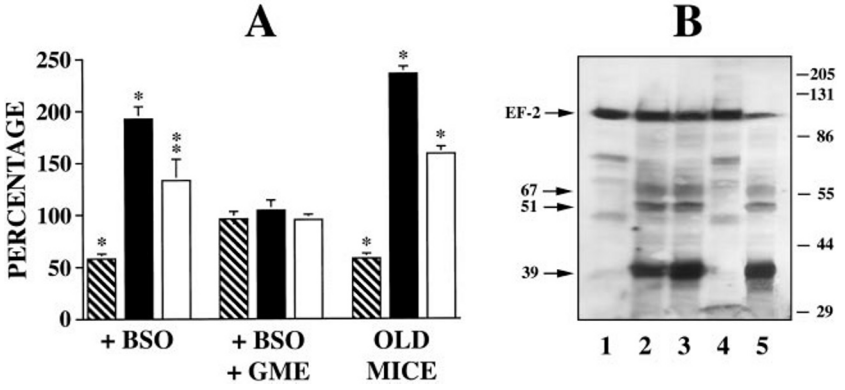

FIG. 6. **Oxidative damage of renal MR and EF-2.** *A*, protein carbonylation and ADP-ribosylation of immunopurified EF-2. Thirty-day-old mice were treated for 10 days with BSO or GME and BSO. The carbonyl group content of MR and EF-2 and the ADP-ribosylatable level were measured in immunopurified samples. The same parameters were also measured in immune pellets obtained from untreated 18-month-old mice. *Bars* represent the means ± S.E. ($n = 4$) for ADP-ribosylatable EF-2 content (*hatched bars*), carbonyl groups in EF-2 (*black bars*), and the MR (*white bars*). Results are presented as a percentage of the value measured in untreated 30-day-old animals (23.5 ± 1.8 nmol of MDA/mg protein, and 387 ± 40 nmol and protein carbonyls group/mg protein). Values are significantly different at *, $p < 0.005$, and **, $p < 0.010$. *B*, proteolytic fragmentation of renal EF-2. Proteins from renal cytosol were resolved by SDS-PAGE, and EF-2 was visualized by Western blotting. Conditions are as follows: *1*, cytosol from 30-day-old mice; *2*, cytosol from BSO-treated mice for 3 days; *3*, cytosol from BSO-treated mice for 10 days; *4*, cytosol from mice co-treated with BSO and GME for 10 days; *5*, cytosol for untreated 18-month-old mice. *Arrows* on the *right side* show molecular weight markers. *Arrows* on the *left side* show the full-length EF-2 band and its main degradation products at 39, 51, and 67 kDa.

described in the liver of rats treated with the radical donor cumene hydroperoxide (25, 34). We emphasize that we were unable to generate an efficient oxidative stress with BSO in adult rats and older mice. BSO is a compound that seems to be efficient to induce oxidative stress only on certain animal models such as guinea pigs, newborn rats, or young mice (48).

Our results provide clear evidence that the impairment of the mineralocorticoid biological response is correlated with an increased content of tissue MDA, a high carbonylation level of MR and EF-2, and a decreased amount of biologically active EF-2 (as determined by its low ADP-ribosylatable level). Interestingly, not all proteins are affected by oxidative stress to an equivalent extent. Thus, the concentration of hsp90 was greatly increased, whereas renal β-tubulin remained unchanged after the treatment with BSO (Fig. 2). In a previous work (14), we have also analyzed the activity of several proteins related to the mechanism of action of MR, such as citrate synthase, 11β-hydroxysteroid dehydrogenase, Na^+/H^+ antiport, and Na^+/K^+-ATPase. There were no dramatic changes in any of these proteins. Moreover, the Na^+/K^+-ATPase activity seems to be preserved due to an increased number of active pumps, so that a decreased specific activity of the Na^+/K^+-ATPase pumps was inferred from these observations. Oxidative stress is intrinsically associated with a state of increased turnover of biomolecules induced by elevated rates of reactive oxygen species. However, extensive studies have recently demonstrated that other proteins also increase their activity upon oxidative stress in a significant manner, for example c-Jun NH_2-terminal kinase (49), p70-S6 kinase (50), Akt/PKB (51), glucose-6-phosphate dehydrogenase (52), PDK1 (53), and SGK (54), among many other examples. Interestingly, the last two proteins are related to the mineralocorticoid biological response. Thus, the serum- and glucocorticoid-dependent kinase (SGK) has been linked to the ALDO-dependent mechanism of activation of the epithelial sodium channel (55, 56). In turn, there exists substantial evidence showing that SGK is regulated by the phosphoinositide-dependent kinase 1, PDK1. Therefore, it seems that there also exist several regulatory mechanisms that are simultaneously triggered by oxidative stress, so that such a compensatory regulation attenuates damaging effects like those evidenced here for the MR-mediated biological response and the translation machinery.

On the other hand, aging is related to a decrease in the stress response and the loss of a low redox potential milieu (22, 57). It has been reported recently (25) that oxidative stress induced by xenobiotics decrease protein synthesis, as this effect is due to a decreased efficiency in the elongation step during the translation process. Similar conclusions were reached when aging-related effects were also compared with free radical damage (34). Because the ALDO binding capacity of renal MR de-

creases with aging, and because the similar pattern evidenced in this work between the harmful effects of GSH depletion and aging, we may certainly speculate that the cumulative damage generated along the lifetime by oxidative stress may affect renal cells (and other tissues as well) in a similar manner as that described here for mice treated with BSO. Nonetheless, a key conundrum such as whether or not protein damaging is primary or secondary in aging still remains to be answered.

It is reasonable to state that the extent of any type of oxidative stress must be exacerbated by a decreased efficiency in the natural antioxidant compounds. Investigation of the GSH enzymatic system in patients with chronic renal failure has revealed that the activities of GSH peroxidase and GSH reductase as well as the plasma GSH concentration were significantly reduced (15). Consistent with the predominant synthesis of GSH peroxidase in the renal tubule, a dramatic decrease in plasma enzymatic activity was also observed. Depletion of GSH can also contribute to renal dysfunction because this thiol is not only an efficient radical scavenger but is also an important detoxicant for eliminating different electrophilic compounds of exogenous and endogenous origin via glutathione *S*-transferase activity. Therefore, GSH deficiency may contribute to the accumulation of harmful compounds.

In summary, the molecular findings presented in this work contribute to an emerging picture that shows a decreased renal MR-dependent biological response as a consequence of the depletion of the GSH antioxidant system. This failure is linked to both post-translational modification of the receptor protein and impaired polypeptide synthesis. These findings may be related to the undesired kidney dysfunction observed during normal aging and certain pathological conditions.

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