rapid communication

Urea-associated oxidative stress and Gadd153/CHOP induction

ZHENG ZHANG, XIAO-YAN YANG, AND DAVID M. COHEN

Division of Nephrology, Hypertension, and Clinical Pharmacology and Division of Molecular Medicine, Department of Medicine, Oregon Health Sciences University and the Portland Veterans Affairs Medical Center, Portland, Oregon 97201

Zhang, Zheng, Xiao-Yan Yang, and David M. Cohen. Urea-associated oxidative stress and Gadd153/CHOP induction. Am. J. Physiol. 276 (Renal Physiol. 45): F786–F793, 1999.—Urea treatment (100–300 mM) increased expression of the oxidative stress-responsive transcription factor, Gadd153/CHOP, at the mRNA and protein levels (at ≥4 h) in renal medullary mIMCD3 cells in culture, whereas other solutes did not. Expression of the related protein, CCAAT/enhancer-binding protein (C/EBP-β), was not affected, nor was expression of the sensor of endoplasmic reticulum stress, grp78. Urea modestly increased Gadd153 transcription by reporter gene analysis but failed to influence Gadd153 mRNA stability. Importantly, upregulation of Gadd153 mRNA and protein expression by urea was antioxidant sensitive. Accordingly, urea treatment was associated with oxidative stress, as quantitated by intracellular reduced glutathione content in mIMCD3 cells. In addition, antioxidant treatment partially inhibited the ability of urea to activate transcription of an Egr-1 luciferase reporter gene. Therefore oxidative stress represents a novel solute-signaling pathway in the kidney medulla and, potentially, in other tissues.

METHODS

Cell culture and Northern analysis. Murine renal medullary collecting duct mIMCD3 cells (32) were grown, passaged, and treated with solute as previously described (7). Total cellular RNA was prepared from mIMCD3 monolayers with RNAzol (Life Technologies) according to the manufacturer’s directions. Equal amounts (7 or 10 µg) of total RNA were subjected to MOPS-formaldehyde-1% agarose gel electrophoresis and transferred via capillary action to Nytran membrane. Probes were prepared from gel-purified (QIAEX II Gel Extraction Kit, Qiagen) restriction-digested Gadd153 cDNA and treated with solute as previously described (7). Whole-cell lysates were prepared from confluent, serum-deprived mIMCD3 cells using a detergent-based lysis buffer as described (43). Equal amounts (in µg, quantitated using the DC Protein Quantitation Assay; Bio-Rad) of lysate were subjected to SDS-PAGE and transferred (Novablot; Pharmacia) to polyvinylidene difluoride for Western blotting with anti-Gadd153, anti-grp78, or anti-C/EBP-β polyclonal antibodies according to the manufacturer’s direc-
Intracellular GSH content. Intracellular GSH content was measured using a modification of the protocol of Shang et al. (34). Cell monolayers were trypsinized, pelleted, resuspended in PBS supplemented with glucose (10 mM), divided equally among sterile microcentrifuge tubes (0.5 ml/tube), and equilibrated for 1 h with a 5% CO2 atmosphere in a 37°C tissue culture incubator (CEDCO). Cells were treated with oxidative stressor and incubated for the indicated times. At appropriate intervals, cells were rapidly pelleted and then homogenized on ice in 120 µl of 10% trichloroacetic acid using a disposable pestle (Kontes). After pelleting at 12,000 rpm for 5 min at 4°C, 100 µl of the supernatant was added to 900 µl of 1 M Tris (pH 8.2) and 10 µl of 10 mM DTNB in methanol. Tubes were covered and incubated at 25°C with occasional agitation for 30 min, after which absorbance at 420 nm was determined. Standards (0–80 µM) were prepared from commercial GSH (Sigma).

RESULTS

Urea specifically increases Gadd153 mRNA and protein expression in renal medullary cells. Cells derived from the murine terminal inner medullary collecting duct (mIMCD3) were treated with hypertonic NaCl (100 mM, 200 mosmol/kgH2O) or supplemental urea (200 mM) for up to 8 h (Fig. 1). Hypertonic NaCl exhibited no effect upon Gadd153 mRNA expression, whereas urea treatment produced a marked increase in mRNA abundance (greater than 10-fold) commencing at 4 h (Fig. 1, top). To confirm that increased Gadd153 mRNA expression was also associated with enhanced Gadd153 protein expression, detergent lysates were prepared from urea- and NaCl-treated cells and subjected to immunoblot analysis with a commercially available anti-Gadd153 polyclonal antibody (Santa Cruz Laboratories; Fig. 2). Under basal conditions, Gadd153 protein expression was undetectable and was unchanged by NaCl treatment (200 mosmol/kgH2O). With urea treatment (200 mM), a marked increase in Gadd153 immunoreactivity was detected. To determine whether this finding was specific for Gadd153, the effect of these solutes upon expression of another C/EBP family member and Gadd153 heterodimerization partner (12), C/EBP-α, was examined (Fig. 2, bottom). In mIMCD3 cells, C/EBP-α was constitutively expressed and highly expressed and was unaffected by either solute. In addition, based upon immunoprecipitation analyses, the ability of Gadd153 to heterodimerize with C/EBP-α was unchanged by treatment with either urea or NaCl (data not shown). To determine the dose dependence of the urea effect, cells were exposed to increasing concentrations of urea for 6 h (Fig. 3A). The effect was maximal at 200 mM urea, absent at 400 mM urea, and intermediate at 300 mM urea (data not shown).
shown). In the depicted exposure, induction at 100 mM urea is also evident. When cells were treated with other permeant (e.g., glycerol) or impermeant (e.g., raffinose or NaCl) solutes at 200 mosmol/kgH2O, there was no demonstrable Gadd153 induction (Fig. 3A). While the present article was in preparation, Kultz and coworkers (25) reported induction of Gadd153 protein expression at 24 h by hypertonic stressors. A longer time course was therefore examined in the present context. Gadd153 was modestly upregulated at 24 h by NaCl (200 mosmol/kgH2O; Fig. 3B), but markedly upregulated at 6, 24, and 48 h by urea treatment (200 mM).

Urea-inducible Gadd153 expression is antioxidant sensitive. Because Gadd153 expression often occurs in the context of oxidative stress, the ability of the sulfhydryl group-containing antioxidant, N-acetylcysteine (NAC), to abrogate the effect of urea was examined (Fig. 4). Cadmium chloride (100 µM) was used as a positive control for oxidative stress in these studies. In the absence of NAC pretreatment, both urea and cadmium chloride increased Gadd153 protein expression at 6 h of treatment. Thirty-minute pretreatment with 30 mM NAC completely inhibited the effect of both urea and cadmium chloride upon Gadd153 expression. To control for a possible nonspecific “conditioning” effect of the modestly hyperosmotic antioxidant, cells were also pretreated for an equal interval with 30 mosmol/kgH2O NaCl. This sham treatment did not influence urea- or cadmium chloride-inducible Gadd153 expression (Fig. 4). Urea-inducible Gadd153 protein expression was also completely inhibited by 30-min pretreatment with another thiol-containing antioxidant, dimethylthiourea (25 mM; data not shown). The effect of NAC upon urea-inducible Gadd153 mRNA abundance was also evaluated. For this purpose, a sensitive RNase protection assay for murine Gadd153 mRNA was developed (see below). NAC markedly suppressed urea-inducible Gadd153 mRNA expression (relative to actin mRNA expression; Fig. 5), whereas it exhibited a very minimal effect under control conditions.
Urea as an inducer of oxidative stress. To confirm that urea was functioning as an oxidative stressor, the effect of urea (200 mM) and hydrogen peroxide (1 mM; a positive control) upon total cellular GSH content as an index of cellular redox state was examined through a colorimetric assay (34). Both urea and peroxide reproducibly decreased intracellular GSH stores in mIMCD3 cells (Fig. 6), consistent with a pro-oxidant effect. In pilot experiments, peroxide (1 mM) decreased GSH content to an extent comparable to that of cadmium chloride (100 µM; data not shown).

To determine the molecular basis for urea-inducible oxidative stress, serial dilutions of potential contaminants of urea and potential byproducts of urea synthesis were examined for their ability to activate Gadd153 expression (Fig. 7). Urea spontaneously isomerizes in aqueous solution to ammonium and cyanate (11); cyanate has been detected in plasma of human end-stage renal disease (ESRD) patients (24, 29, 30). Carbamylated proteins result from the interaction of cyanate with protein amino groups (reviewed in Ref. 37). Ammonium (10 µM to 1 mM; Fig. 7A), cyanate (10 µM to 10 mM; Fig. 7B), and carbamate (1 µM to 10 mM; Fig. 7C) ions at concentrations up to 10 mM (Fig. 7) did not reproduce the urea effect. Of note, as seen in Fig. 7, nonspecific bands (unchanged in intensity by any condition examined) migrating faster and slower than the band representing Gadd153 were inconsistently detected. The earlier observation that cyanate generation from urea proceeds as a function of time and temperature (11) prompted a series of experiments looking at the effect of these parameters upon urea inducibility of Gadd153. Incubation of fresh urea stock for 30 min at 95°C and for 24 h at 37°C (conditions that markedly accelerated cyanate generation; Ref. 11) produced no detectable increment in Gadd153-inducibility (data not shown). Furthermore, the possibility of heavy metal contamination was excluded by the inability of pretreatment of the urea stock solution with the heavy metal-chelating resin, Chelex, to block the effect of urea upon Gadd153 mRNA expression (data not shown). Urea of maximal purity (e.g., electrophoresis grade) from three separate suppliers was examined; all sources produced equivalent results.

Transcriptional activation of Gadd153. To determine the extent to which transcriptional activation accounted for the observed increase in Gadd153 mRNA, mIMCD3 cells were transiently transfected with a luciferase reporter gene driven by the 5′-flanking sequence of the human GADD153 gene (18). The included sequences were sufficient to confer transcriptional inducibility in diverse models through the cellular injury response element (17) and AP-1 site (19). Urea only very modestly (but reproducibly) increased GADD153 transcription by 35% at 6 h of treatment (Fig. 8A). Potent activators in other systems similarly exhibited only modest induction in the present model. The oxidative stressor cadmium chloride increased transcription by 46%, whereas the alkylating and DNA-damaging agent, methyl methanesulfonate (MMS), exerted no effect. These data suggested that, in the mIMCD3 cell model, additional posttranscriptional regulatory processes were potentially playing a role in Gadd153 mRNA and protein expression. Alternatively, additional regulatory sequences absent from the available promoter fragment may have been required for maximal inducibility in this model.

Effect of urea upon GRP78 expression. Conditions associated with increased Gadd153 expression are also associated with increased expression of the molecular chaperone, grp78, as a consequence of stress in the endoplasmic reticulum (42). Immunoblot analyses of lysates prepared from mIMCD3 cells subjected to the same stressors described above were performed with anti-grp78 antibody and subjected to densitometric quantitation (Fig. 8B). Urea (200 mM for 6 h) exhibited no effect upon grp78 abundance, whereas cadmium chloride and MMS increased grp78 protein abundance at 6 h by 46% and 96%, respectively.
Effect of urea upon Gadd153 mRNA stability. Because of the modest effect of urea upon Gadd153 transcription, the ability of urea to increase Gadd153 mRNA half-life was examined in detail. Basal Gadd153 mRNA expression was inconsistently detected using Northern analysis (Fig. 1); therefore decay curves under control conditions would have been difficult to generate. We established an RNase protection assay for murine Gadd153 to address this question. A murine Gadd153 cDNA fragment was PCR generated and used to prepare nonisotopically labeled Gadd153 riboprobe. Of note, actinomycin D, the transcriptional inhibitor used for these studies, does not appreciably influence Gadd153 mRNA stability (22). Consistent with Northern analyses (Fig. 1), Gadd153 mRNA abundance exceeded control by at least fourfold (Fig. 9). Under control conditions, half-life of Gadd153 mRNA was in excess of 2 h and remained unchanged in the presence of urea treatment. As an additional control, following 6 h of urea treatment, cells were removed from hyperosmotic urea and Gadd153 mRNA decay was observed in the absence of actinomycin D treatment. Under these circumstances, the half-life was again similar to control (data not shown). Therefore it was unlikely that increased Gadd153 mRNA stability contributed to the increase in Gadd153 mRNA abundance in response to urea treatment.

Effect of NAC upon urea-inducible Egr-1 reporter gene activity. Because intracellular signaling through reactive oxygen intermediates has been observed in other contexts (see below), the role of oxidative stress in urea signaling to immediate early gene transcription...
was examined. We have previously shown that urea activates Egr-1 transcription to a much greater extent than any other solute examined (8). Consistent with our prior observations, urea (200 mM for 6 h) increased transcription of a luciferase reporter gene driven by 1.2 kb of the 5'-flanking sequence (38) of the murine Egr-1 gene over sevenfold (Fig. 10). Pretreatment of control-treated mIMCD3 monolayers with NAC produced a modest decrease (28%) in luciferase activity, whereas NAC pretreatment markedly decreased urea-inducible luciferase activity by 55%. These data were consistent with a role for oxidative stress signaling in urea-inducible immediate-early gene transcription.

**DISCUSSION**

Signaling via oxidative stress constitutes a new model of solute signaling in the renal medulla and, potentially, a novel model of systemic urea signaling operative in clinical uremia. Urea has previously been shown to activate transcription of a subset of transcription factor-encoding immediate-early genes (6, 8). Urea inducibility of these genes is conferred by serum response elements and adjacent clusters of Ets motifs in their 5'-flanking sequences (8). These composite DNA elements bind the serum response factor and the transcriptional activation is conferred by MEK-dependent ERK activation and subsequent Elk-1 phosphorylation (5). Urea also activates other signaling events characteristic of receptor tyrosine kinase-mediated signaling, including phosphorylation of phospholipase C-γ and generation of IP3 (8).

On the basis of chemical structure alone, urea would not be expected to function as an oxidative stressor. Nonetheless, the ability of the NAC and other thiol-containing antioxidants to block urea-inducible Gadd153 expression, in conjunction with the ability of urea to lower the intracellular concentration of GSH, suggests that this is a signaling event activated in a specific fashion in response to urea treatment. No other hyperosmotic solute examined, whether permeant or impermeant, upregulated Gadd153 expression in this time frame (see below). Three potential contaminants and catabolites of urea did not reproduce the effect. Oxidative stress was not contributed by a contaminating heavy metal, because Chelex treatment did not eliminate the effect. The change in intracellular redox state was fairly rapid, paralleling that occurring in response to the addition of hydrogen peroxide. While this report was in preparation, Kultz and coworkers (25) recently reported that prolonged exposure (24-48 h) of medullary cells to hypertonic stress (NaCl) resulted in upregulation of Gadd153 expression at the protein level. Only at 24 h was a modest increase in Gadd153 protein expression demonstrable in the present studies, whereas the effect of urea was pronounced and persisted to 48 h.

Urea signaling bears multiple hallmarks of a receptor tyrosine kinase-mediated event (7). Precedents exist for signaling via reactive oxygen intermediates in response to stimuli known to activate receptor tyrosine kinases (1, 21, 35). In addition, exogenous peroxide may potentiate tyrosine phosphorylation of receptor tyrosine kinases (16, 23). Therefore intracellular oxidative stress may represent a physiological component of urea signaling and an adaptive response to hyperosmotic urea in the kidney medulla. This view is supported by the ability of NAC to partially abrogate the effect of urea upon signaling to immediate-early gene transcription (Fig. 10).

The specific role of Gadd153 expression in the present context is unknown. As a transcription factor, Gadd153 regulates expression of downstream genes (39, 41). Transactivation by Gadd153 is regulated in some models by the hypertonicity-responsive p38 MAPK (40), which some investigators have also reported to be significantly urea responsive in renal medullary cells (3). These observations suggest a model through which NaCl and urea may synergize to activate transcription in a Gadd153-dependent fashion in cells of the renal medulla. Urea-inducible Gadd153 expression accompanied by NaCl-inducible p38 action in the renal medulla (3, 44) could induce a simultaneous increase in Gadd153 abundance and Gadd153 posttranslational modification. With respect to a role for Gadd153 function in vivo, in one rodent model, the endoplasmic reticulum stressor, tunicamycin, induced reversible acute renal failure and concomitant Gadd153 (CHOP) upregulation in the proximal tubule. Interestingly, in chp −/− mice, the histological evidence of renal injury in response to tunicamycin is markedly attenuated (45). The clinical course of the acute renal failure, however, remains essentially unchanged. Importantly, c/ebpβ −/− mice, lacking the principal heterodimerization partner for Gadd153, exhibit a phenotype identical to that of the chp −/− mice (45).
Wang et al. (42) observed that grp78 expression was markedly increased by all stimuli that activated Gadd153 expression. Urea, in marked contrast, failed to upregulate grp78 expression (Fig. 8). This discrepancy could be explained by dissimilar signaling pathways activated by urea with respect to other examined stresses. For example, urea might not be a bona fide endoplasmic reticulum stressor. Consistent with this possibility, urea failed to activate transcription from the UPRE in transient transfection reporter gene assays (data not shown). In addition, a non-UPRE-dependent mitogenic-like pathway of (constitutive) grp78 expression has recently been described (44). An alternative explanation concerns the dissimilar models employed; specifically, the potent activators of grp78 expression, cadmium chloride and MMS, produced only modest effects upon grp78 expression in the present model. Therefore a more subtle urea-inducible increment in grp78 expression might not be evident or achieve statistical significance.

The marked increase in Gadd153 mRNA expression detectable at 4 h of urea treatment, in conjunction with the relatively modest (less than twofold) increase in Gadd153 transcription (in reporter gene assay), suggested one of two possibilities: 1) Gadd153 mRNA accumulation in cells of the renal medulla may require, in part, urea-inducible mRNA stabilization; or 2) urea-inducible transcriptional activation of Gadd153 in medullary cells requires additional sequence information beyond that which is present in the available reporter gene construct. RNase protection assay was performed to address the former question in quantitative fashion. Under both control and urea-treated conditions, Gadd153 mRNA half-life exceeded 2 h and was unlikely to contribute to the observed increase in mRNA abundance. Through the use of actinomycin D, Jackman et al. (22) determined the half-life of Gadd153 mRNA to be ~1 h in exponentially growing cells and ~2 h in the presence of medium depletion or growth arrest. The latter more closely resemble the serum-deprived confluent culture conditions examined in the present model. Gadd153 mRNA half-life may be increased to greater than 4 h by several DNA-damaging stressors (22); however, transcriptional activation of the Gadd153 gene accounts overwhelmingly for the increased abundance of Gadd153 mRNA under similar conditions. Previously, at least two consensus motifs, the AP-1 site (9) and the cellular injury response element, containing an Sp1 site (18), have been shown to mediate inducible Gadd153 transcription in reporter gene analyses. Both were present within the promoter fragment (~786-TATA-Luc) examined for the present studies. A search for additional upstream enhancer elements potentially responsive to urea in the Gadd153 5’-flanking sequence is underway. The positive controls examined for the reporter gene assay, MMS and cadmium chloride, are known to increase Gadd153 mRNA abundance in a largely transcription-dependent fashion, yet even these treatments failed to markedly upregulate Gadd153 reporter gene activity in the mIMCD3 cell model. An additional explanation for this apparent discrepancy is suggested by the case of Gadd45, wherein a family of closely related proteins exhibiting both differential tissue distribution and differential stress responsiveness at the mRNA level has recently been described (36). It is possible that the urea-responsive Gadd153-like protein, which exhibits anti-Gadd153 immunoreactivity and whose mRNA permits Gadd153 cDNA hybridization, represents a product of an additional Gadd153-like gene. Review of the Gadd153/CHOP-10-homologous murine-expressed sequence tags (more than 20 different sequences) in the IMAGE Consortium database supports the plausibility of this interpretation (data not shown).

Lastly, a potential relationship between urea and systemic oxidative stress (in contrast to medullary oxidative stress), although not directly supported by the present data, can be inferred. Atherosclerosis and malignancy are the principal causes of mortality in patients with ESRD (15, 26), and oxidative stress has been broadly implicated in the pathogenesis of both (10, 27). The uremic state is associated with biochemical indexes of increased oxidative stress (2), the genesis of which remains obscure. Uremia is defined by a markedly elevated circulating concentration of urea that fluctuates rapidly with the initiation of effective dialytic therapy. Urea is readily membrane permeant, so the increase in circulating urea concentration is not confined to the intravascular space. In light of the present findings, it is conceivable that at least a portion of the enhanced oxidative stress associated with uremia could be mediated by urea itself. The molecular level at which this effect is mediated, however, remains speculative.

In summary, we have demonstrated the unique ability of urea to activate expression of Gadd153 in an antioxidant-sensitive fashion. Consistent with this finding, urea treatment induced oxidative stress. These data define a new urea signaling pathway operative in renal medullary cells and suggest the possibility of systemic urea signaling through oxidative stress in clinical uremia.

We thank Nikki Holbrook for the Gadd153 cDNA and Steven B. Howell for the Gadd-Luc (~786-TATA-Luc) reporter. This work was supported by a Grant-in-Aid from the American Heart Association and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-54924.

Address for reprint requests and other correspondence: D. M. Cohen, PP262, OHSU, 3314 S.W. US Veterans Hospital Rd., Portland, OR 97201 (E-mail: cohend@ohsu.edu).

Received 30 October 1998; accepted in final form 18 February 1999.

REFERENCES


4. Brewer, J. W., J. L. Cleveland, and L. M. Hendershot. A pathway distinct from the mammalian unfolded protein response...

Koshishii, I., and T. Imanari.State analysis of endogenous cya-


Wang, X. Z., B. Lawrence, J. W. Brewer, H. Zinszner, A. Sanjay, L. J. Mi, R. Boorstein, G. Kreibich, L. M. Hender-

