Chronic Hyperosmolality Enhances ANP-Dependent cGMP Production via Stimulation of Transcription and Protein Synthesis in Cultured Rat IMCD Cells

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KUSANO, E., IIMURA, O., ISHIDA, F., AKIMOTO, T., AMEMIYA, M., ANDO, Y. and ASANO, Y. *Chronic Hyperosmolality Enhances ANP-Dependent cGMP Production via Stimulation of Transcription and Protein Synthesis in Cultured Rat IMCD Cells.* Tohoku J. Exp. Med., 2002, **197** (4), 209-220 — Recently, we found that hyperosmolality acutely inhibited atrial natriuretic peptide (ANP) dependent cGMP production by reducing ANP binding sites in cultured rat inner medullary collecting duct (IMCD) cells. Therefore, the present study was undertaken to evaluate the chronic effect of hyperosmolality on ANP dependent cGMP production in IMCD cells. Cell culture was carried out either in an iso-osmotic or hyperosmotic solution consisting of equi-isomolar NaCl and/or urea. Incubations with ANP and/or other agents were performed under the same osmotic conditions. ANP or SNP stimulated cGMP production was enhanced in a chronically hyperosmotic medium. These changes occurred in an osmolality-dependent manner. Hyperosmolality with sodium alone or with sodium and urea, but not with urea alone, was effective for the enhancement of ANP action. There was no significant difference between ¹²⁵I-ANP specific bindings under iso-osmotic and hyperosmotic conditions. Incubation with cytoskeleton modulators did not affect ANP-dependent cGMP production stimulated by hyperosmolality. On the other hand, both actinomycin D, an inhibitor of transcription, and cycloheximide, an inhibitor of protein synthesis, prevented the stimulatory effects of hyperosmolality. The results suggest that chronic hyperosmolality enhances ANP-dependent cGMP production via stimulation of transcription and protein synthesis in cultured rat IMCD cells. — hyperosmolality; ANP; cGMP; inner medullary collecting duct cells; protein synthesis

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Atrial natriuretic peptide (ANP) acts on several sites in the kidney and stimulates intracellular cyclic guanosine monophosphate (cGMP) production as a major second messenger (Takeda et al. 1986; Light et al. 1989). The inner medullary collecting duct (IMCD) is one of the major target sites of ANP (Light et al. 1989), and this nephron segment is important for final regulation of urine production. The renal medulla is unique in that its osmolality rises dramatically even under physiologic conditions. In rats, the medullary osmolality can be as high as 3000 mOsm/kgH₂O in severe dehydration (Beck and Byung 1982). However, the precise mechanism by which hyperosmolality affects hormone action, including that of ANP, in the IMCD is not clear. With respect to the action of ANP, Berl et al. (1992) reported an increase in ANP-induced cGMP production in rat IMCD cells after long term (48 hours) culture in a hyperosmotic medium with Na ions. On the other hand, in the membrane fraction prepared from rabbit renal inner medullary cells, Gunning et al. (1989) reported that the activity of ANP-sensitive particulate guanylate cyclase was suppressed by short-time incubation with hypertonic medium. In microdissected IMCD and glomeruli, short term incubation with a hyperosmotic medium with Na and urea suppressed the ANP sensitive cGMP production (Shinohara et al. 1994). We recently found that short-term incubation in a hyperosmotic medium with Na, urea or mannitol inhibited ANP-dependent cGMP production by reducing the number of ANP binding sites in cultured rat IMCD cells (Iimura et al. 1995). These studies suggest that the ANP action might be modulated differently either by short-term or long-term hyperosmolality in IMCD cells. Thus, we investigated the effect and the mechanism of long-term incubation with hyperosmolality on ANP-dependent cGMP production in cultured IMCD cells.

MATERIALS AND METHODS

Culture of rat IMCD cells

IMCD cells were obtained from renal papillae of 150–200 g male Sprague-Dawley rats as previously described (Iimura et al. 1995; Oono et al. 1996). Briefly, the papillary tissue was finely minced with a razor blade under sterile conditions. Minced tissues were transferred into a 10-ml culture medium (1:1 mixture of DMEM, Ham's F-12 buffered with 10 mM HEPES at pH 7.35, 5 mg/ml transferrin and 5 mg/ml human insulin, 50 nM hydrocortisone, 5 pM triiodothyronine, 29.0 mEq/l NaHCO₃, 50 IU/ml penicillin, 50 mg/ml streptomycin) containing 10 mg collagenase and 10 mg bovine albumin. After incubation at 37°C in 5% CO₂ atmosphere for 90 minutes, 20 ml of distilled water was added to lyse cells other than collecting duct cells by osmotic shock. After tubes were centrifuged at 900 rpm for 5 minutes, the supernatant was discarded and the pellet was resuspended in phosphate-buffered saline (PBS) containing 50 IU/ml penicillin and 50 mg/ml streptomycin. After centrifugation at 900 rpm for 5 minutes, the pellet was resuspended in the culture medium with 10% fetal bovine serum (FBS) and harvested in a 24-well culture dish. Cells were incubated at 37°C in 5% CO₂ atmosphere. After 24 hours, the medium was changed to that containing 1% FBS. All studies were performed on the fourth day of culture in a confluent state.

Experimental procedures

Three days after changing the medium with 1% FBS, the cells were incubated with either an iso-osmotic or hyperosmotic medium (450 mOsm/KgH₂O) consisting of equi-osmolar NaCl and urea. After 24-hours incubation with test agents such as phorbol 12-myristate 13-acetate (PMA), cytochalasin-B, colchicine, cycloheximide and actinomycin D, cells were rinsed with either the iso-osmotic Krebs-Ringer buffer (KRB) at pH 7.35 or hyperosmotic KRB,

and incubated with 100 nM ANP (Peptide Institute Inc., Osaka) for 5 minutes in iso-osmotic or hyperosmotic KRB. Hyperosmotic solutions were made by adding NaCl and/or urea to the iso-osmotic KRB or the culture medium. In each experiment, the phosphodiesterase inhibitor 3-isobutyl-1 methyl xanthine (MIX, Sigma) was added to the final incubation solution at a concentration of 0.5 mM. Subsequent, the medium was aspirated and the cells were immediately immersed in 0.2 ml of 0.1 N HCl to stop the reaction. Cells were then collected into glass tubes and boiled for 3 minutes. After 15-minute centrifugation at 6500 rpm, the supernatant was decanted and 0.05 ml of 50 mM sodium acetate was added for the assay of cGMP. The pellet was used for protein measurements.

Measurement of intracellular cGMP contents

cGMP and protein contents of each sample were assayed by the enzymeimmunoassay (cGMP EIA assay kit, Amersham, Bucks, UK) and by the method of Lowry (1951), respectively. Then, the cGMP content was normalized to the protein content of each sample. The lower limit of detection in cGMP was 2 fmol/sample.

Receptor binding assay

A receptor binding assay was performed by using the methods previously described (Iimura et al. 1995). Briefly, cells were preincubated with the iso-osmotic or hyperosmotic culture medium at 37°C for 24 hours. Each well was washed four times with 0.5 ml of ice cold culture medium of the same composition. The cells were then incubated for 1 hour at 4°C with the iso-osmotic or hyperosmotic culture medium containing ^{125}I -ANP(1-28) (from New England Nuclear, Boston, MA, USA) and 0.2% bovine serum albumin. This incubation period was chosen since the binding of ^{125}I -ANP was found to be saturated within 1 hour at 4°C (Specific binding at 1 hour was about 90% of that at 12 hours incubation.). Then the cells were washed

four times with 0.5 ml of ice-cold solution and solubilized with 0.5 ml of 0.1 N NaOH. Radioactivity was counted in a γ -counter (ARC-600, Aloka, Tokyo). Nonspecific binding was determined in the presence of 1 μM unlabeled ANP and was subtracted from the total binding.

Statistics

The results are expressed as mean $\pm$ S.E. (number of samples). Data were analyzed by analysis of variance combined with Scheffe multiple comparisons or unpaired *t*-test, as appropriate. Differences with $p < 0.05$ were considered to be significant. Each figure is a representative one of at least three separate experiments.

RESULTS

Time course of ANP-stimulated cGMP production with hyperosmotic medium

As was the case in our previous study (Iimura et al. 1995), short-term incubation (<6 hours) with hyperosmotic medium inhibited ANP-dependent cGMP production, while long-term incubation (>24 hours) enhanced ANP action (Fig. 1). Twenty-four-hours incubation maximized ANP-dependent cGMP production. On the other hand, the iso-osmotic medium did not affect ANP-dependent cGMP production at all. Thus, in the following experiments, the long-term stimulatory effect of hyperosmolality was exclusively examined with 24 hours incubation.

Effect of chronic hyperosmolality on SNP-stimulated cGMP production

To check whether the stimulatory effect of hyperosmolality is specific for ANP action, we also examined the effect of hyperosmolality on sodium nitroprussid (SNP)-dependent cGMP production. It is well known that ANP binds to surface receptors coupled to particulate guanylate cyclase, whereas SNP activates soluble guanylate cyclase. Again, hyperosmolality caused enhancement of cGMP pro-

Fig. 1. Time course of ANP-stimulated cGMP production with medium hyperosmolality. After preincubation in an iso-osmotic (○: 300 mOsm/Kg H₂O) or hyperosmotic (●: 600 mOsm/Kg H₂O, 1:1 of NaCl and urea) medium, ANP 10⁻⁷ M and 3-isobutyl-1 methyl xanthine 5 × 10⁻⁴ M were added for 5 minutes to stimulate cGMP production. Values are means ± S.E. (n = 6). **p* < 0.05 (vs. time 0), #*p* < 0.05 (vs. corresponding iso-osmotic condition).

duction induced by 10⁻⁴ M SNP (Fig. 2). Therefore, hyperosmolality activates not only particulates but also soluble guanylate cyclase in IMCD cells.

Effect of chronic hyperosmolality with either NaCl or NaCl plus urea or urea alone on ANP-stimulated cGMP production

To determine whether this enhancement by chronic hyperosmolality on ANP action depends on osmolytes, we tested several different combinations of osmolytes, i.e., NaCl, NaCl plus urea, and urea alone to produce hyperosmolality. Hyperosmolality with NaCl, NaCl plus urea, but not urea alone, were effective for the enhancement of ANP-stimulated cGMP production. In addition, these effects occurred in an osmolality-dependent manner (Figs. 3A, 3B and 3C). Therefore, in the subsequent experiments, we used the medium of 450 mOsm/Kg H₂O with NaCl plus urea as a hyperosmotic solution.

Effect of chronic hyperosmolality on ANP-specific binding in IMCD cells

To investigate the mechanisms involved in the enhancement of cGMP production, at first, specific binding of ¹²⁵I-labelled ANP to IMCD was measured, since we previously reported that acute hyperosmolality rapidly and markedly reduced ANP binding to IMCD. As shown in Fig. 4, there was no significant difference between ¹²⁵I-ANP specific bindings under iso-osmotic and hypertosmotic conditions.

Effect of cytoskeleton modulators on chronic hyperosmolality-induced upregulation of ANP-dependent cGMP production

To examine whether the cytoskeleton system, which has been reported to alter receptor kinetics, plays a role in the stimulatory effect of hyperosmolality, we pretreated cells with cytoskeleton modulators such as cytochalasin-B (100 μM) and colchicine (50 μM). However, they did not alter the stimulatory effect of hyperosmolality at all (Fig. 5).

Fig. 2. Effect of chronic hyperosmolality on SNP-stimulated cGMP production.

After 24-hours preincubation in an iso-osmotic (300 mOsm/Kg H₂O) or hyperosmotic (600 mOsm/Kg H₂O, 1 : 1 of NaCl and urea) medium, SNP 10⁻⁴ M and 3-isobutyl-1 methyl xanthine 5 × 10⁻⁴ M were added for 5 minutes to stimulate cGMP production. Values are means ± s.e. (n = 4). *p < 0.01 (vs. 300).

Effect of actinomycin D or cycloheximide on chronic hyperosmolality-induced upregulation of ANP-dependent cGMP production

Finally, to examine the involvement of transcription or protein synthesis in the stimulatory action of hyperosmolality, we investigated the effects of actinomycin D or cycloheximide on ANP-dependent cGMP production. Actinomycin D is known as a transcription inhibitor. Although actinomycin D itself stimulated ANP-induced cGMP production, this compound prevented the stimulatory effect of hyperosmolality at concentrations of more than 1 µg/ml (Fig. 6). On the other hand, cycloheximide, a protein synthesis inhibitor, also prevented the stimulatory effect of hyperosmolality at concentrations of more than 2 µg/ml (Fig. 7). These results indicate that both DNA transcription and protein synthesis may be involved in the enhancement of ANP-dependent cGMP production induced by hyperosmolality.

DISCUSSION

In the present study, we demonstrated that chronic hyperosmolality upregulated not only ANP- but also SNP-stimulated cGMP production. Chronic hyperosmolality with NaCl and NaCl plus urea, but not urea alone, were effective for the enhancement of ANP-stimulated cGMP production. With respect to the mechanism, hyperosmolality did not affect ANP binding. Cytoskeleton modulators also did not affect the stimulatory effect of hyperosmolality on ANP action. However, actinomycin D, an inhibitor of transcription, and cycloheximide, an inhibitor of protein synthesis, prevented the stimulatory effects of hyperosmolality. These findings indicate that chronic hyperosmolality upregulates ANP-dependent cGMP production via both transcription and protein synthesis in cultured rat IMCD cells.

IMCD is one of the targets of various hormones, such as ANP, AVP and endothelin, and this nephron segment is important for the final regulation of urine production (Light et al.

Fig. 3. Effect of chronic hyperosmolality either with NaCl alone (A), NaCl plus urea (B), or urea alone (C) on ANP stimulated cGMP production. After 24-hours preincubation either in an iso-osmotic (300 mOsm/Kg H₂O) or hyperosmotic medium with Na alone, Na plus urea, or urea alone, ANP 10^{-7} M and 3-isobutyl-1 methyl xanthine 5×10^{-4} M were added for 5 minutes to stimulate cGMP production. The hyperosmotic medium was 450 or 600 mOsm/Kg H₂O with the addition of 75 mM or 150 mM of NaCl (A). The hyperosmotic medium was 450 or 600 mOsm/Kg H₂O with the addition of 1 : 1 of NaCl and urea (B). The hyperosmotic medium was 450 or 600 mOsm/Kg H₂O with the addition of urea 150 mM or 300 mM (C). Values are means $\pm$ S.E. ($n=6$). ^{*} $p < 0.05$ (vs. corresponding 300), ^{**} $p < 0.01$ (vs. corresponding 300).

Fig. 4. Effect of chronic hyperosmolality on ANP specific binding in IMCD cells.

After 24-hours preincubation in an iso-osmotic (300 mOsm/Kg H₂O) or hyperosmotic (450 mOsm/Kg H₂O; 1:1 of NaCl and urea) medium, cells were incubated in a medium containing 2 nM ¹²⁵I ANP for 60 minutes at 4°C. Nonspecific binding was determined in the presence of 1 μM unlabeled ANP and was subtracted from the total binding. Binding assay was performed with the medium adjusted to the corresponding osmolality. Values are means ± S.E. (n = 8).

1989; Oono et al. 1996). In the rat IMCD, ambient osmolality can vary from less than 300 to over 3000 mOsm/kgH₂O even under physiological conditions. Thus, it is conceivable that the response of this segment to these hormones is affected by extracellular osmolality. With respect to ANP action, we and other investigators have reported that acute hyperosmolality inhibited ANP-dependent cGMP production, while chronic hyperosmolality enhanced ANP action in this nephron segment (Gunning et al. 1989; Berl et al. 1992; Shinohara et al. 1994; Iimura et al. 1995). However, the precise mechanisms for chronic hyperosmolality on ANP action are not clear at the present time.

As for the stimulatory effect of chronic hyperosmolality on ANP-stimulated cGMP production, Berl et al. (1992) demonstrated that osmolality induced by sodium alone or sodium

and urea, but not urea alone, were effective for the enhancement of ANP action. This was also confirmed by the results of the present study. Although sodium ions are permeable, Na, K-ATPase pump them out from the cells, thereby contributing to the medium hyperosmolality. In contrast, urea is highly permeable in the rat IMCD, and it raises the osmolality acutely but does not keep the hyperosmolality chronically. Therefore, it is reasonable to expect that urea would exert inhibitory effect of ANP action in an acute hyperosmotic condition but it would not affect ANP action in a chronic hyperosmotic condition. However, further study is required to elucidate whether the intracellular Na ion concentration or hypertonicity per se affects ANP-dependent cGMP production in a chronic hyperosmotic condition.

It is known that receptor kinetics is

Fig. 5. Effects of cytochalasin-B and colchicine on ANP-dependent cGMP production induced by chronic hyperosmolality. After 24-hours preincubation in an iso-osmotic (300 mOsm/Kg H₂O) or hyperosmotic (450 mOsm/Kg H₂O; 1:1 of NaCl and urea) medium, incubation with cytochalasin-B 10^{-4} M for 20 minutes or with colchicine 5×10^{-5} M for 60 minutes was performed. Then ANP 10^{-7} M and 3-isobutyl-1 methyl xanthine 5×10^{-4} M were added for 5 minutes to stimulate cGMP production. Solid columns indicate preincubation in an iso-osmotic medium (300 mOsm/kg H₂O) and the hatched columns indicate preincubation in a hyperosmotic medium (450 mOsm/kg H₂O). Values are means $\pm$ S.E. ($n=7$). * $p < 0.05$ (vs. corresponding iso-osmotic preincubation).

influenced by ambient osmolality. In LLC-PK1 cells, Lutz and Kumar (1993) reported that enhancement of the AVP-stimulated cAMP production in a hypertonic medium may be due to an inhibition of receptor endocytosis. On the other hand, Williams et al. (1992) reported glucose-induced down-regulation of AVP receptors in cultured rat aortic smooth muscle cells. With respect to ANP receptors, Katafuchi et al. (1992, 1993) showed that an increase in NaCl concentration of the medium resulted in a dramatic decrease in the number of type C ANP receptor binding sites in cultured bovine endothelial cells. In water-deprived rats, Kollenda et al. (1990) reported that C type ANP receptor density on the glomerular membrane increased, while that of B type receptor remained unchanged. Ballermann et al. (1985) demonstrated that glomerular ANP receptor

density was reduced in rats fed a high-salt diet. While a significant reduction of the ANP binding to the IMCD cells appears to be an important mechanism responsible for the acute inhibitory effect of hyperosmolality on the basis of the results of our previous study (Iimura et al. 1995), the enhancement of ANP binding to its receptor does not seem to be the mechanism for the upregulation of ANP-stimulated cGMP production with chronic hyperosmolality on the basis of the results of the present study.

Several lines of evidence suggest that the cytoskeleton organization is altered by an osmotic change and that such a change has an important role in the control of cell function (Foskett and Spring 1985; Bencherif and Lukas 1993; Kapeller et al. 1993). In a human clonal cell line expressing acetylcholine receptors, Bencherif and Lukas (1993) showed that cyto-

Fig. 6. Effect of actinomycin D on ANP-dependent cGMP production induced by chronic hyperosmolality. After cells were preincubated either in an iso-osmotic (300 mOsm/Kg H₂O) or hyperosmotic (450 mOsm/Kg H₂O; 1:1 of NaCl and urea) medium with (1~25 µg/ml) or without actinomycin D for 24 hours, ANP 10⁻⁷ M and 3-isobutyl-1 methyl xanthine 5 × 10⁻⁴ M were added for 5 minutes to stimulate cGMP production. Solid columns indicate preincubation in an iso-osmotic medium (300 mOsm/kg H₂O). Hatched columns indicate preincubation in a hyperosmotic medium (450 mOsm/kg H₂O). Values are means ± s.e. (n = 4). *p < 0.01 (vs. corresponding iso-osmotic condition), #p < 0.05 (vs. corresponding Actinomycin D 0).

chalcasin treatment induced marked changes in the numbers and distribution of acetylcholine receptors. Therefore, it was interesting to investigate whether the cytoskeleton plays a role in changing the receptor kinetics in the present study. However, preincubation of the cells with colchicine or cytochalasin-B, which would disrupt microtubules and microfilaments respectively, did not affect the stimulatory effect of hyperosmolality. Thus, it is unlikely that the cytoskeleton system is greatly involved in the enhancement of ANP-dependent cGMP production in a chronically hyperosmotic condition.

Actinomycin D is known to inhibit DNA transcription and mRNA synthesis by binding to the guanosine residues. In the presence of actinomycin D, the enhancement of ANP-dependent cGMP production was eliminated in

the present study. However, actinomycin D alone increased ANP-dependent cGMP production in a dose dependent fashion both in the conditions of iso-osmolality and hyperosmolality. Since we used a nonspecific inhibitor of phosphodiesterase, 3-isobutyl-1 methyl xanthine, the increase of ANP-dependent cGMP production exclusively reflects the increase of guanylate cyclase activity. Although actinomycin D is considered not to affect the post-transcriptional step, i.e., mRNA degradation, several lines of evidence support the view that transcriptional blockage increases stability of certain mRNAs through the redistribution from nucleus to cytoplasm of pre-existing mRNA-stabilizing proteins (Nakielnny and Dreyfuss 1997). However, whether the increase in ANP stimulated cGMP production by actinomycin D correlates with redistribution

Fig. 7. Effect of cycloheximide on ANP-dependent cGMP production induced by chronic hyperosmolality. After cells were preincubated either in an iso-osmotic (300 mOsm/Kg H₂O) or hyperosmotic (450 mOsm/Kg H₂O; 1:1 of NaCl and urea) medium with (2~50 μg/ml) or without cycloheximide for 24 hours, ANP 10⁻⁷ M and 3-isobutyl-1 methyl xanthine 5 × 10⁻⁴ M were added for 5 minutes to stimulate cGMP production. Solid columns indicate preincubation in an iso-osmotic medium (300 mOsm/kg H₂O). Hatched columns indicate preincubation in a hyperosmotic medium (450 mOsm/kg H₂O). Values are means ± s.e. (n = 4). *p < 0.002 (vs. corresponding iso-osmotic condition).

ogf mRNA-stabilizing proteins in IMCD cells remains to be determined.

Since a protein synthesis inhibitor, cycloheximide, prevented the enhancement of ANP-dependent cGMP production induced by chronic hyperosmolality, de novo synthesis of protein could be involved in this mechanism. MAP kinase (Itoh et al. 1994; Terada et al. 1994) and MAP kinase-kinase (Terada et al. 1994) and the early gene transcription factors such as Egr-1 and c-fos are known to be upregulated in MDCK cells exposed to a hypertonic condition (Cohen et al. 1991). However, it is not yet clarified whether de novo synthesis of these factors contributes to the hyperosmolality-induced upregulation of ANP-dependent cGMP production in the IMCD. In the present study, chronic hyperosmolality enhanced not only ANP-stimulated cGMP production but also SNP-stimulated cGMP production. This means that hyperosmolality stimulates not only particulate guanylate cyclase but also

soluble guanylate cyclase activity. However, as for the mechanism of the stimulatory action of hyperosmolality on soluble guanylate cyclase, we do not know whether the same mechanism as the particulate guanylate cyclase may operate or not from the present study.

In summary, chronic hyperosmolality mainly induced by sodium ions enhanced ANP-dependent cGMP production in cultured rat IMCD cells. The increase in ANP-stimulated cGMP production appeared to be mediated, at least in part, by the stimulation of DNA transcription and de novo synthesis of particulate guanylate cyclase. A long-term hyperosmotic condition may promote ANP-induced sodium chloride excretion to prevent further hyperosmolality in the renal papillary interstitium.

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