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Ammonium Activates Ouabain-Activated Signalling Pathway in Astrocytes: Therapeutic Potential of Ouabain Antagonist

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Abstract: The causal role of ammonium in hepatic encephalopathy was identified in 1930s. Astroglial cells are primary cellular elements of hepatic encephalopathy which conceptually, can be considered a toxic astrogliopathology. Previously we have reported that acute exposure to ammonium activated ouabain/Na,K-ATPase signalling pathway, which includes Src, EGF receptor, Raf, Ras, MEK and $ERK_{1/2}$. Chronic incubation of astrocytes with ammonium increased production of endogenous ouabain-like compound. Ouabain antagonist canrenone abolished effects of ammonium on astrocytic swelling, ROS production, and upregulation of gene expression and function of TRPC1 and $Ca_v1.2$. However, ammonium induces multiple pathological modifications in astrocytes, and some of them may be not related to this signalling pathway. In this review, we focus on the effect of ammonium on ouabain/Na,K-ATPase signalling pathway and its involvement in ammonium-induced ROS production, cell swelling and aberration of Ca^{2+} signals in astrocytes. We also briefly discuss Na,K-ATPase, EGF receptor, endogenous ouabain and ouabain antagonist.

Keywords: Ammonium, astrocytes, canrenone, Na,K-ATPase, ouabain.

1. INTRODUCTION

Hyperammonemia resulting from urea cycle deficiencies, Reye's syndrome or liver failure causes polymorphic mental and behavioral manifestations such as confusion, forgetfulness, irritability as well as alterations of consciousness represented by lethargy, somnolence and, in the terminal stages, coma associated with brain oedema that causes death [1, 2]. Brain oedema is linked to delayed stimulation of NKCC1, a cotransporter of Na^+, K^+, $2Cl^-$ and water (for review, see [3]). The causal role of ammonium in hepatic encephalopathy was identified in 1930s [4] and it is now generally accepted that characteristic symptomatology in experimental fulminant hepatic failure results from an increase in brain concentration of ammonium to 3 - 5 mM [5]. In hepatic encephalopathy and acute liver failure these concentrations are generally lower [6].

Astroglial cells are primary cellular targets of hepatic encephalopathy, and the astroglia-specific glutamine synthetase [7] is a central enzyme providing for ammonium detoxification [8-10]. Increased activity of glutamine synthetase in the presence of elevated ammonium in the brain affects major homeostatic functions of astroglia, and fundamentally hepatic encephalopathy can be considered a toxic astrogliopathy.

Our previous studies identified enhanced Na,K-ATPase activity in cultured mouse astrocytes exposed to chronic treatment with ammonium [11]. This was linked to an increase in expression of α_2 subunit of Na,K-ATPase (NKA), which arguably results from activation of a signalling cascade comprised of Src, EGF receptor, Raf, Ras, MEK and $ERK_{1/2}$. Phosphorylation of the $ERK_{1/2}$ induces up-regulation of the α_2 gene expression [12, 13]. Incidentally, this signalling sequence is almost identical to the pathway initiated by a low concentration of ouabain in a kidney cell line [14]. The ouabain antagonist, canrenone is able to abolish ammonium-induced astrocytic swelling [13]. Recently, we have found that canrenone also inhibits upregulation of gene expression and function of TRPC1 and $Ca_v1.2$ in astrocytes after chronic treatment with 3 mM ammonium [15, 16]. In this paper, we will focus on ouabain signal pathway and its relevance in ammonium pathology in astrocytes.

2. ENDOGENOUS DIGITALIS

Endogenous digitalis, also known as endogenous cardiotonic steroids (CTS) were discovered in 1990s [17]. These compounds contain steroid structure and have inhibitory effect on Na/K-ATPase at high concentrations, but stimulate ouabain/Na,K-ATPase signalling pathway at very low concentrations. There are two classes of endogenous digitalis, endogenous cardenolides and endogenous bufadienolides.

2.1. Endogenous Cardenolides

Endogenous cardenolides are represented by endogenous ouabain and endogenous digoxin. Their chemical structures are identical to plant-derived compounds. Endogenous ouabain was the first endogenous cardenolide found in human serum [17]. The adrenal medulla and hypothalamus contain high level of endogenous ouabain [18-20]. The role

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of endogenous ouabain in human hypertension has been established [21]. The level of endogenous ouabain was increased in amygdala, hippocampus and hypothalamus in response to salt-loading in rats [22]. Endogenous digoxin was purified from human urine in 1990 [23]. It remains controversial whether endogenous digoxin is an antagonist of endogenous ouabain (for review, see [24]).

2.2. Endogenous Bufadienolides

Bufadienolides are different from cardenolides by a doubly unsaturated six-membered lactone ring in their structure. Marinobufagenin (MBG) in mammalian plasma and urine was identified by using different techniques, including immunoassays and mass spectrometry. Similarly to endogenous ouabain, the role of MBG in hypertension has been extensively studied (for review, see [24]). Telocinobufagin is another bufadienolide, which may be a precursor of MBG [25].

2.3. Synthesis of Endogenous Digitalis in Brain

On account of their steroidal structure, biosynthesis of endogenous digitalis was studied in adrenocortical tissue or in endocrine cells according to the classic scheme of steroidgenesis. Cholesterol is converted to pregnenolone catalyzed by the cytochrome P450 side chain cleavage (P450 scc), and pregnenolone is converted to progesterone catalyzed by the 3β-hydroxy-steroid dehydrogenase (3β-HSD). The enzymes converting progesterone into endogenous digitalis are unknown [24]. Bufadienolides are derived from cholesterol, but cholesterol side chain cleavage is probably not involved.

In the brain, steroidogenesis seems to occur mainly in astrocytes. Astrocytes express P450 scc and 3β-HSD, as well as other enzymes involved in steroidogenesis [26]. Astrocytes in culture produce pregnenolone, progesterone and, other neurosteroids [27, 28]. Astrocytes also express steroid acute regulating protein (StAR) and peripheral-type benzodiazepine receptor (PBR), which transfer cholesterol into mitochrondrial [29-31]. Upregulation of PBR was detected in human hyperammonemic disorders, in experimental hyperammonemic syndromes and in astrocytes exposed to ammonium *in vitro* (for review, see [32]). In addition, PBR agonists induce mitochrondria swelling, oxidative damage and steroidogenesis [32]. In pour previous studies, we had found that incubation of astrocytes with 3 mM ammonia for 4 days increased an endogenous compound with ouabain-like activity by 50% [11]. The released endogenous “ouabain” during 4 days reached 3.7 μg/mg protein in control (part of which, in principle might originate from the serum added to the incubation media), and 5.4 μg/mg in ammonium-treated cells, which was a significantly increase.

3. OUABAIN SIGNALLING PATHWAY

3.1. Na,K-ATPase

Na/K-ATPase might be a primary target for ammonium toxicity due to similarities between K^+ and NH_4^+ [33]. Ammonium increases Na/K-ATPase activity in cultured mouse astrocytes due to the enhanced production of ouabain-like compounds [11]. The Na/K-ATPase is composed of two essential subunits, α and β. The α subunits are catalytic, they span the membrane multiple times and contain the binding sites for Na^+, K^+, ATP and the specific inhibitor ouabain and thus also the ouabain antagonist canrenone [34]. The β subunit is a single span glycoprotein with most of its mass exposed to the extracellular space [35]. There are four isoforms of α subunit, namely α_1, α_2, α_3 and α_4. In adult brain and in cultured CNS cells, the α_1 isoform is expressed in both neurones and astrocytes, α_2 is a virtually astrocyte-specific isoform, and α_3 is only expressed in neurones [36, 37]. The α_1 isoform also functions as a receptor ligand for signalling, mediated by nanomolar concentrations of ouabain or endogenous ouabain-like compounds.

3.2. EGF Receptor (EGFR)

The activation of EGFRs activates two major intracellular signalling cascades, represented by the MAPK/ERK and PI3K/AKT pathways. EGF can induce phosphorylation of all five known tyrosine phosphorylation sites of EGFR [38]. $EGFR^{Y992}$, $EGFR^{Y1173}$ and $EGFR^{Y1045}$ are autophosphorylation sites, with $EGFR^{Y1173}$ being the major one and $EGFR^{Y992}$ being the minor one. $EGFR^{Y845}$ is known to be the major Src phosphorylation site [39-41]. $EGFR^{Y1068}$ is not phosphorylated in the brain *in vivo* [42] and in cultured astrocytes, unless stimulated by EGF addition [38] or following production of an EGFR ligand, as indicated by its phosphorylation by ammonium treatment, which stimulates EGFR (Fig. **1**).

3.3. Ouabain Signalling Pathway

The ouabain signalling pathway has been well established in kidney cell lines. Binding of ouabain to α_1 isoform recruits Src which in turn phosphorylates EGFR and initiates its conventional intracellular signalling pathways, MAPK/ERK and PI3K/AKT (Fig. **1**) [43]. This process is independent of shedding of growth factor(s) and at least partly occurs in lipid rafts, where it depends on the presence of caveolin, the major component of the lipid raft [44].

The Na/K-ATPase/ouabain signalling pathway is involved in the intracellular signalling of ammonium in primary cultures of astrocytes. Ammonium-induced Na/K-ATPase/Src/EGFR interaction occurs instantly. A study by Dai *et al.* [13] shows that twenty minutes of incubation with 3 mM ammonium induced an increase of phosphorylation at Y845 and Y1068 of EGF receptor. The phosphorylation levels at Y992, Y1045 and Y1173 were however unchanged. Ammonium induced EGFR activation can be inhibited by the EGFR inhibitor AG1478 and Src inhibitor PP1, but not by zinc-dependent metalloproteinase GM6001, indicating that ammonium induced EGFR activation is ligand-independent [13]. The process of interaction induced by ammonium among α_1 isoform, Src, EGF receptor, $ERK_{1/2}$, AKT (Fig. **1**) and caveolin-1 occurs in lipid raft. Crosstalk between MAPK/ERK and PI3K/AKT induced by ammonium is shown by inhibition of ammonium-induced phosphorylation of $ERK_{1/2}$ by both the MAPK inhibitor U0126, and the AKT inhibitor LY294002. This confirms that PI3K and AKT are likely to operate upstream of $MAPK/ERK_{1/2}$ [45]. Canrenone, a ouabain antagonist can inhibit ERK phosphorylation induced by ammonium [13].

In cardiac cells ouabain can induce production of reactive oxygen species, ROS (Fig. **1**) (for review, [46]). The

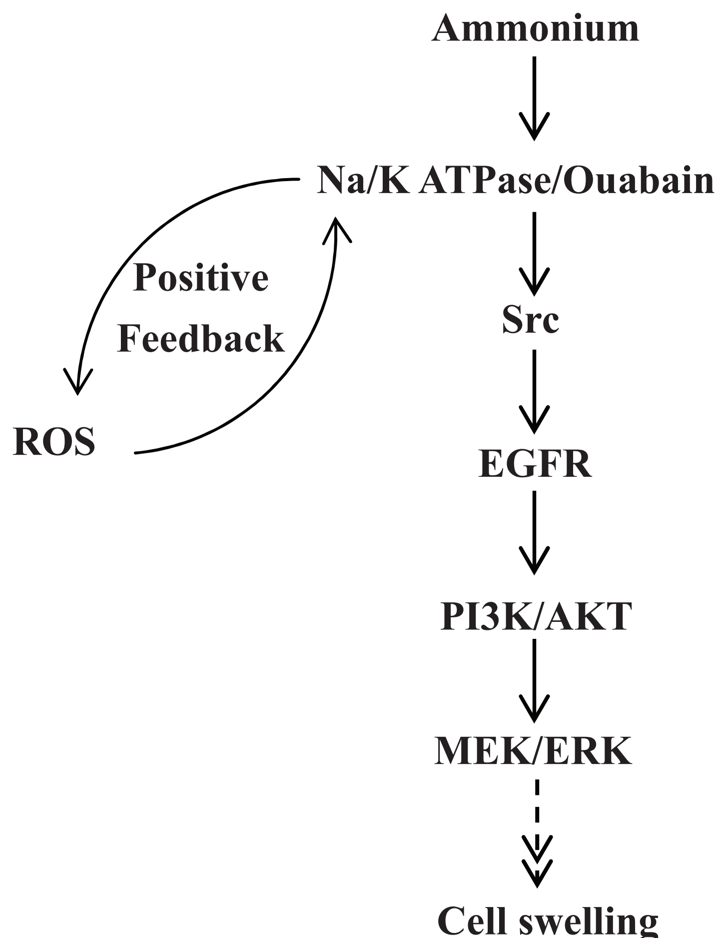

Fig. (1). Diagram showing signal pathways for EGFR transactivation in response to 3 mM NH_4Cl. Ammonium acts on the Na,K-ATPase to activate both its activity and Na,K-ATPase/ouabain signalling. The latter proceeds *via* Src to the EGF receptor (EGFR). We have previously shown that this occurs *via* MEK and therefore probably also *via* Ras and Raf [70]. We now have shown that also AKT needs to be activated upstream of ERK, but where this occurs in relation to Ras and Raf is not known with certainty. We have also previously shown that ERK phosphorylation can lead to NKCC1 activation, but not determined the intermediate steps [47]. The swelling caused by 3 mM ammonium is delayed, beginning after 12 hr, a delay caused by the requirement for ROS and its action on NKCC1. Since we have shown that ROS does not operate directly *via* the EGF receptor it is suggested that ouabain-mediated ROS stimulation, confirmed in the present study, in a feed-forward reaction re-inforces the effect of ouabain, as suggested by Liu *et al*. [46]. Literature evidence suggests that higher concentrations of ammonium may also act directly on NKCC1. Such concentrations are generally not encountered in hepatic encephalopathy, but occur in other hypeammonemic conditions. From Dai *et al*. [13].

involvement of ROS in ammonium-induced $ERK_{1/2}$ phosphorylation in astrocytes is indicated by the inhibitory effect on the phosphorylation by superoxide dismutase (SOD), an inhibitor of free radicals (Fig. **2A**). In contrast with the effect of ammonium, H_2O_2-induced $ERK_{1/2}$ phosphorylation in astrocytes can not be inhibited by AG 1478, indicating that this effect is not mediated *via* the EGF receptor (Fig. **2B**). Nevertheless, the observation that the effects of ouabain on ammonium-induced ERK phosphorylation, ROS production and cell swelling all can be inhibited by an ouabain antagonist indicates that enhanced ouabain signalling is responsible for all three phenomena [13]. This raises the question how ROS can increase brain swelling in the absence of any effect on the EGFR, which is involved in the pathway activating NKCC1 [47]. A possible answer to this question is a suggested feed-back increase of Na,K-ATPase signalling by ROS [46]. As illustrated in Fig. **1**, the well established increase in ROS production resulting from ouabain signalling may therefore further increase Na,K-ATPase/ouabain signalling and all its down-stream effects. This time-consuming sensitizing process probably explains why ammonium-induced edema in most cases is delayed as discussed below.

4. EFFECTS OF OUABAIN INHIBITOR

4.1. Cell Swelling

Astrocytic swelling that occurs in hepatic brain edema and can be induced by administration of ammonium into primary cultures of astrocytes [48, 49]. Ammonium-induced astrocytic swelling develops slowly, and swelling did not become apparent until 8 h of exposure. The swelling could be completely prevented by EGF receptor inhibitor AG1478, the Src inhibitor PP1, the MAPK inhibitor U0126 and the

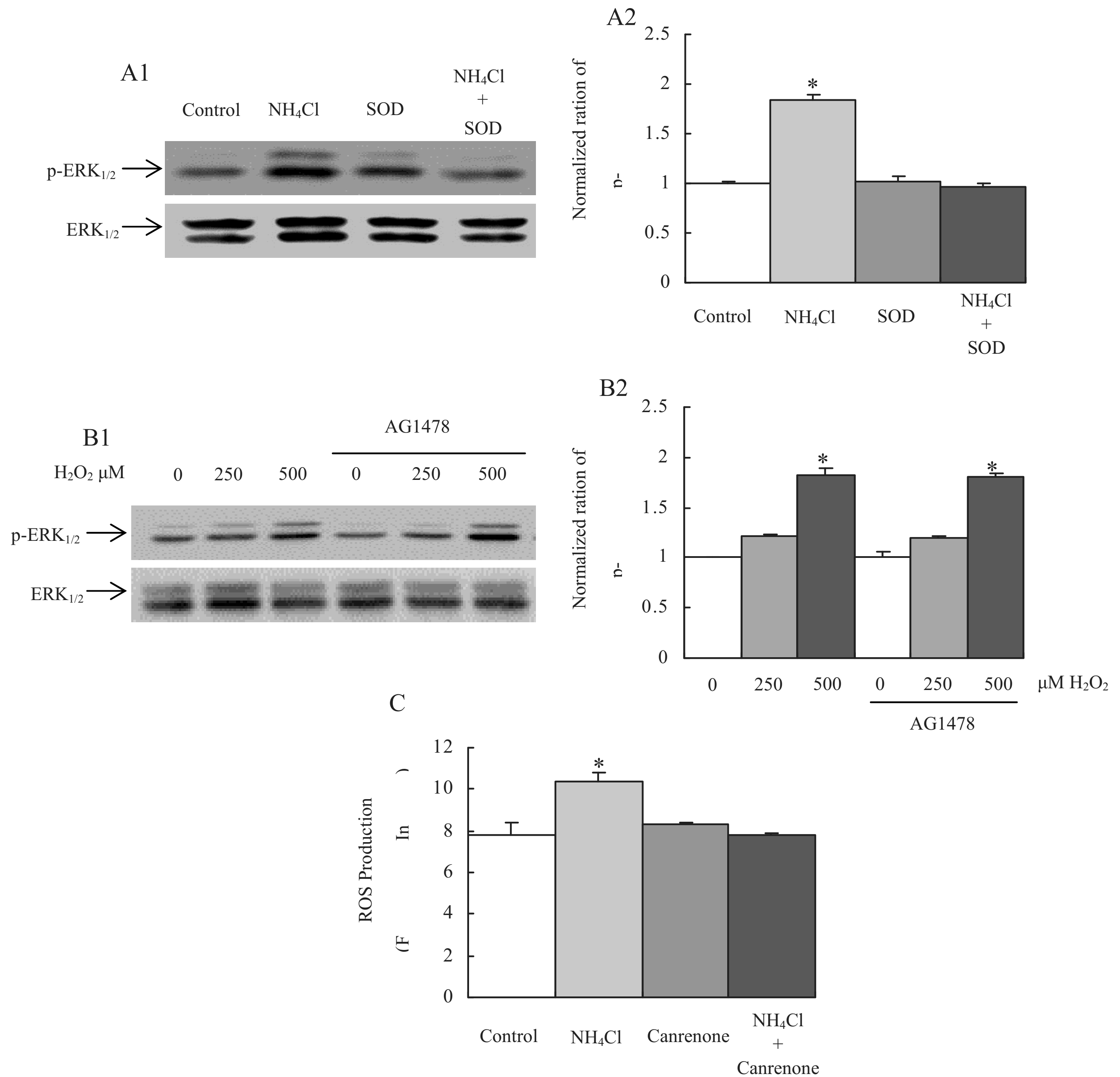

Fig. (2). Ammonium -induced $ERK_{1/2}$ phosphorylation can be inhibited by SOD, and ROS production can be inhibited by canrenone, but H_2O_2-induced $ERK_{1/2}$ phosphorylation does not require EGFR activity. (**A**) Cells were incubated with 0 or 3 mM NH_4Cl in the absence or presence of 25 U/ml SOD for 2 h. (**B**) Cells were incubated with 0, 250 or 500 μM H_2O_2 in the absence or presence of 1 μM AG1478 for 8 min. Thereafter, cells were harvested, and the level of $ERK_{1/2}$ phosphorylation was measured by Western blotting. (**A1** and **B1**) Immunoblots from a representative experiment. Similar results were obtained from three independent experiments. (**A2** and **B2**) Average ERK phosphorylation was quantitated as ratios between p-$ERK_{1/2}$ and $ERK_{1/2}$. The ratio between p-$ERK_{1/2}$ and $ERK_{1/2}$ in control group was designated a value of one. (**A2**) *Statistically significant (P<0.05) different from all other groups. (**B2**) *Statistically significant (P<0.05) different from 0 and 250 μM H_2O_2 with or without AG1478 groups. D Song, H Dai and L Peng, unpublished results. (**C**) Ammonium-induced ROS production. Cells were incubated with 0 or 3 mM NH_4Cl in the absence or presence of 100 μM canrenone for 2 h. ROS was determined as fluorescence intensity of oxidized carboxy-H_2DCFDA. *Statistically significant (*P*<0.05) different from all other groups. From Dai *et al*. [13].

AKT inhibitor LY294002 [13], indicating the role for ouabain/Na,K-ATPase signalling pathway. The mechanisms of the astrocytic swelling are not clear. However, ammonium can replace K^+ in stimulation of Na,K-ATPase activity in mammals [50-53], and Na,K-ATPase activity provides the driving force for NKCC1 [54].

4.2. Aberrat Ca^{2+} Signals

Up-regulation of TRPC1: Treatment of cultured astrocytes with ammonium for 3 days increased expression of TRPC1 mRNA in a concentration-dependent manner; with 1 mM NH_4^+ showing no effect, whereas 3 and 5 mM

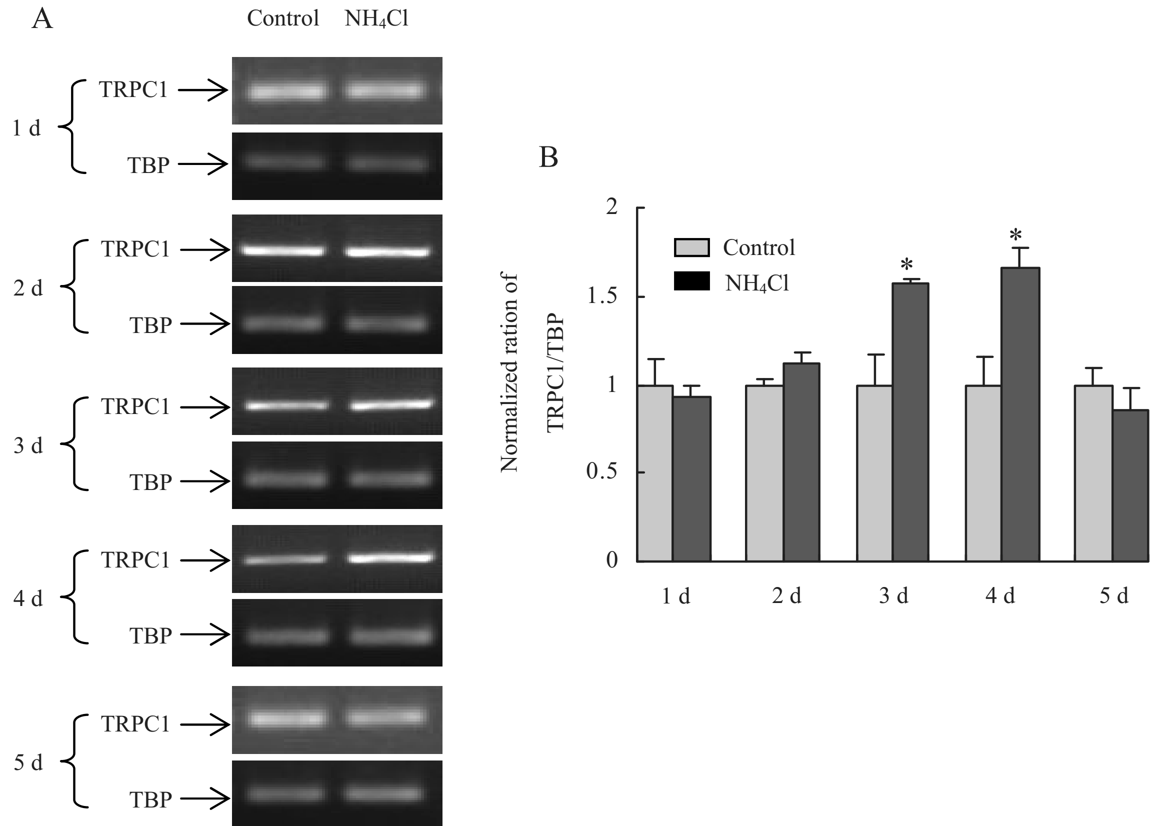

Fig. (3). Up-regulation expression of TRPC1 mRNA and protein in primary cultures of mouse astrocytes chronically treated with ammonium. Cells were treated with PBS (Control) or with 3 mM NH_4Cl for 1-5 days. Thereafter, cells were harvested, and mRNA expression was measured by RT-PCR. (**A**) Southern blot from a representative experiment. Similar results were obtained from three independent experiments. (**B**) Average mRNA expression was quantified as a ratio between TRPC1 and TBP, as a housekeeping gene. *Statistically significant ($P<0.05$) different from all other groups. T Du, F Wang and L Peng, unpublished results.

causing a significant upregulation of mRNA presence [15]. The highest level of expression was observed at 3 mM being 180 ± 9.5% of control group. Changes in expression of TRPC1 protein expression mirrored those of mRNA [15]. The increase in mRNA became significant after 3 and 4 days of treatment and declined after 5 days of treatment (Fig. **3**). In addition, $[Ca^{2+}]_i$ responses to the β-adrenergic agonist isoproterenol, the α_2-adrenergic agonist dexmedetomidine, the $InsP_3$ receptor ($InsP_3R$) agonist adenophostin A and ryanodine receptor (RyR) agonist 4- chloro-m-cresol (4-CMC) are significantly increased in astrocytes chronically exposed to ammonium [15]. Similarly, the store-operated Ca^{2+} entry (SOCE) meditated by the transient receptor potential channel 1 (TRPC1), is significantly augmented. Increases in TRPC1 expression and in SOCE were both prevented by the ouabain antagonist canrenone [15]. Up-regulation of TRPC1 gene expression was also found in the brain of adult mice treated with intraperitoneal injections of urease for 3 days and in astrocytes, but not in neurones freshly isolated from similarly treated transgenic mice tagged with an astrocyte-specific or a neurone-specific markers [15].

Up-regulation of $Ca_v1.2$: The ammonium-induced increase in Ca^{2+} influx in astrocytes resulted from an up-regulation of $Ca_v1.2$ channels expression identified at mRNA and protein levels (T Du, F Wang and L. Peng, unpublished results). Increase in $Ca_v1.2$ expression was also prevented by ouabain antagonist canrenone (T Du, F Wang and L. Peng, unpublished results).

Astrocytes are non-excitable neural cells that utilise dynamic fluctuations of intracellular Ca^{2+} and Na^+ concentrations for their excitability [55]. Regulated release of Ca^{2+} from endoplasmic reticulum (ER) through intracellular Ca^{2+} release channels, such as the $InsP_3$ receptors ($InsP_3R$) and ryanodine receptors (RyRs) (for review, see [55, 56]) have a leading role in glial Ca^{2+} signalling. According to contemporary views, SOCE can be mediated either by ORAI channels that mediate calcium release activated Ca^{2+} current (I_{CRAC}) or by some members of the extended family of transient receptor potential (TRP) cationic channels [57-59]. In astrocytes the major and essential component of SOCE is represented by "canonical" TRPC1 channels [60]. Another source of increase in $[Ca^{2+}]_i$ are astroglial L-type calcium channels. We have demonstrated that activation of these channels results in a relatively modest Ca^{2+} entry, which however triggers substantial $[Ca^{2+}]_i$ increases due to a consequent Ca^{2+}-induced Ca^{2+} release (CICR) form the ER mediated by RyRs [61, 62]. Ammonium-induced deregulation of astroglial Ca^{2+} signalling should be considered as an important pathogenic step, which in turn may trigger gene expressions, contribute to cell damage, mitochondrial

permeability transition (MPT) and increased glutamate exocytosis. Ammonium-induced aberration of Ca^{2+} homeostasis and Ca^{2+} signalling can define the disease progression, and its potential inhibition might be used for specific therapy.

4.3. Ouabain Inhibitors

The prodrug of canrenone, spironolactone is the first antialdosteronic drugs developed in 1960s. It is used for the treatment of hypertension, primary hyperaldosteronism and peripheral oedema related to the cardiac failure and other pathologies associated with aldosteronism (for review, [63]). Spironolactone is not well tolerated since it also binds to progesterone and androgen receptors. Canrenone competes with ouabain for the same binding site, and reverses the inhibition of Na,K-ATPase by ouabain and ouabain-like factor [64, 65]. It has also been reported that canrenone has anti-MBG effect [65]. After i.v. infusion of the water soluble derivative of spironolactone, ^{3}H-canrenoate-K, ^{3}H-activity in white matter of the brain was 36X and in brain cortex 27X the activity in plasma [66]. In addition, canrenone accounted for 72% of the total concentration in CSF. This finding suggests canrenoate exerts pharmacological effects on the CNS and can be used in hyperammonemia in clinic.

Rostafuroxin (PST 2238) is derived from digistoxigenin. This coumpound antagonizes all ouabain functional effects by specifically displacing ouabain binding from the high affinity Na,K-ATPase isoform present in the caveolae [67]. Digibind is a commercial preparation containing the purified Fab fragment of the sheep antidigoxin antibody, which cross-reacts with other cardiotonic steroids.

5. CONCLUSION

We have suggested that 3 mM ammoniun, a concentration relevant for cell culture studies of hepatic encephalopathy, exerts ouabain-like effects on Na,K-ATPase's signalling, which lead to EGFR transactivation, PI3K/AKT and MAPK/ERK phosphorylation and ROS formation. This could possibly result from the ability of ammonium to stimulate the Na,K-ATPase in the same manner as K^{+}. Although astrocytes synthesize endogenous ouabain, it is unlikely that ouabain concentration would increase fast enough to have an immediate effect. However, cerebral ammonium toxicity is undoubtedly multifactorial. At least some of ammonium effects are probably not related to ouabain signalling, such as ammonium-induced increase in glutamine formation from glutamate [68]. Some ammonium effects (reduction of pyruvate/lactate ratio) are due to a resulting decrease in glutamate and can in cultured astrocytes be prevented by addition of excess glutamate [69]. On the other hand prevention of glutamine production by inhibition of glutamine synthetase can for unknown reasons reduce or prevent edema formation [reviewed in 33] in the brain *in vivo*, where the situation may be even more complicated. Experiments are in progress to evaluate potential therapeutic effects of canrenone during ammonium toxicity in animal experiments.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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