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Original Contribution

Reactive oxygen species are physiological mediators of the noradrenergic signaling pathway in the mouse supraoptic nucleus

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ABSTRACT

Free radicals are essential for the vasopressin (AVP) response to plasmatic hyperosmolarity. Noradrenergic afferents are the major projections on the supraoptic nucleus (SON) of the hypothalamus and stimulate the expression of AVP via a nitric oxide (NO) pathway. In this study, we investigated the mechanisms linking free radicals and noradrenaline (NA)-induced regulation of AVP. Analysis of Tg8 transgenic mice, invalidated for the monoamine oxidase-A gene and with consequently high levels of brain monoamines and AVP in the SON, showed that free radicals are more abundant in their SON than in that of wild-type mice (WT). Antioxidant superoxide dismutase 1 and 2 and catalase enzyme activities were also higher in these mice than in WT. This may explain the observed absence of cytotoxicity that would otherwise be associated with such high level of free radicals. Treatment of Tg8 mice with α-MPT, a blocking agent for NA synthesis, decreased both the production of free radicals and the AVP levels in the SON. Furthermore, incubation of ex vivo slices including the SON with NA increased the production of free radicals and AVP levels in wild-type mice. When NA was associated with α-lipoic acid, an antioxidant blocking the production of free radicals, AVP remained at its control level, indicating that free radicals are required for the effect of NA on the expression of AVP. In slices incubated with SNP, a producer of NO, free radicals and AVP levels increased. When NA was associated with L-NAME (a NO synthase blocker), the levels of free radicals and AVP were the same as in controls. Thus, the noradrenaline–NO pathway, which stimulates the expression of vasopressin, involves free radicals. This study provides further evidence of the physiological importance of free radicals, which should no longer be considered solely as cytotoxic factors.

Free radicals, or reactive oxygen species (ROS)[2], are mostly described as molecules involved in pathological processes such as cancer and neurodegenerative diseases [1–3]. However, recent work suggests that ROS act also as signaling molecules in the normal cell environment and that they stimulate transcription factors involved in the regulation of various physiological states: they have been associated with the control of feeding [4–6], melanocortin tone [7], and synaptic plasticity and memory [8,9]. In the context of osmoregulation, we have previously shown that ROS production in the supraoptic nucleus (SON) of the hypothalamus is necessary for the stimulation of arginine vasopressin (AVP) expression and release by the hypothalamoneurohypophyseal complex to normalize plasmatic osmolality when the osmotic axis is activated [10].

AVP is synthesized by the magnocellular neurons of the SON, which are primarily innervated by noradrenergic inputs from the A1/A6 cell groups of the brain stem [11–13]. Both electrophysiological and pharmacological studies implicate the noradrenergic system in the regulation of AVP release [14,15] via the activation of α- and β-adrenoreceptors [16–18]. The effect of noradrenaline (NA) on vasopressinergic neurons is mediated by complex interactions involving glutamate, GABA, galanin, astrocytes, the extracellular matrix, and nitric oxide (NO) [18–23]. Because NO may interfere with the production of ROS [24,25], we asked whether ROS take part in the noradrenergic regulation of AVP expression within the SON. We also analyzed the involvement of free radicals relative to NO in this pathway.

Abbreviations: aCSF, artificial cerebrospinal fluid; ALA, α-lipoic acid; AVP, arginine vasopressin; COMT, catecholamine *O*-methyltransferase; DHE, dihydroethidine; H_2DCFDA, dichlorofluorescein diacetate; ip, intraperitoneally; L-NAME, l-arginine methyl ester; NA, noradrenaline; Nox, NADPH oxidase; MAO, monoamine oxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; α-MPT, α-methylparatyrosine.

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For this purpose, we first used the transgenic mouse model Tg8, invalidated for the monoamine oxidase A (MAO-A) gene, which results in elevated levels of brain monoamines, including NA [26], and increased AVP expression and release in the hypothalamoneurohypophyseal system [15,21]. Analysis of this mouse line demonstrated that free radical concentrations and expression of antioxidant enzymes in the SON are higher than in wild-type mice. We then used an ex vivo experimental model, based on hypothalamic slices from male C3H/HeJ mice, to demonstrate that ROS are necessary for NA stimulation of AVP expression. Therefore, we show for the first time that ROS are necessary in the NA–NO–AVP pathway in the SON.

Materials and methods

Animals

All the experiments were in accordance with French and European law (Decree 87–848, 86/609/ECC). Six-week-old male C3H/HeJ (Janvier breeding, France) or Tg8 (homozygous and wild-type) mice [26] were used. They were housed under a 12 h light, 12 h dark cycle at 20 ± 2 °C and provided with food and water ad libitum.

Experiments were done in triplicate with four (histochemical experiments) or eight (biochemical experiments) mice in each experimental group.

α-Methylparatyrosine (α-MPT) treatment

Tg8 male mice were injected intraperitoneally (ip), once daily for 3 successive days, with α-MPT methyl ester (300 mg/kg; Sigma, France) or a similar volume of vehicle. This α-MPT treatment decreases NA levels by at least 80% in rodents [27] and causes the Tg8 monoaminergic phenotype to revert to a C3H/HeJ phenotype [15,26]. Mice were sacrificed 4 h after the last injection.

Wild-type, Tg8, and α-MPT-treated Tg8 mice were used for AVP and antioxidant enzyme immunohistochemistry and ROS detection.

Slice preparation

After anesthesia (pentobarbital, 25 mg/kg ip), C3H/HeJ mouse brains were quickly removed and immersed in cooled artificial cerebrospinal fluid (aCSF): 117 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose. Coronal hypothalamic slices were cut with a Vibroslice (World Precision Instruments, UK). With the optic chiasma as a landmark, one 400-μm-thick section including the SON was selected for each mouse, transferred to a brain slice chamber system, and equilibrated in aCSF for 1 h. Viability of the slices was controlled for by trypan blue staining and their histological preservation by cresyl violet staining as previously described [20,23,28]. Slices were then subjected to pharmacological test conditions.

Drug application

NA treatment

After equilibration, slices were incubated for 45 min, 1 h, or 2 h with 10^{-4} M NA (Sigma) dissolved in 0.01% ascorbic acid in aCSF [23,29]. We chose this NA concentration with reference to previous electrophysiological studies conducted on brain slices, in which application of 10^{-4} M NA induced an optimal response from magnocellular neurons [29]. Control slices were incubated with 0.01% ascorbic acid in aCSF.

ROS inhibition

After equilibration, hypothalamic slices were pretreated with α-lipoic acid (ALA; 400 μM; Sigma) [30–32] or aCSF (untreated slices) for 45 min and then treated for 45 min with either 0.01% ascorbic acid–aCSF (control-untreated), 0.01% ascorbic acid–aCSF+ALA (control-ALA), 0.01% ascorbic acid–aCSF+10^{-4} M NA (NA-untreated), or 0.01% ascorbic acid–aCSF+10^{-4} M NA+ALA (NA-ALA).

Nitrergic pathway

After equilibration, slices were incubated with 10^{-4} M NA, 0.1 mM sodium nitroprusside (SNP; Sigma), 3 μM nitro-L-arginine methyl ester (L-NAME; Sigma), or L-NAME+NA (3 μM and 10^{-4} M, respectively) for 45 min, all dissolved in 0.01% ascorbic acid–aCSF.

At the end of the pharmacological treatments, slices were processed for AVP immunohistochemistry and ROS detection.

AVP and antioxidant enzyme immunohistochemistry

Mice were anesthetized (pentobarbital, 25 mg/kg ip) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer; brains were removed, frozen, and cut into sections (20 μm thick).

ex vivo slices were fixed overnight at 4 °C with 4% paraformaldehyde in 0.1 M phosphate buffer, cryoprotected, frozen, and sectioned (20 μm thick).

Sections including the SON were blocked by incubation for 1 h in 0.05 M phosphate-buffered saline (PBS), pH 7.4, 1% bovine serum albumin (BSA; Sigma), 0.2% Triton X-100, and then incubated overnight with rabbit antiserum against AVP (1:2000 [33]), Cu/Zn-superoxide dismutase (SOD1, 1:100, Santa Cruz Biotechnology, USA), Mn-superoxide dismutase (SOD2, 1:100, Santa Cruz Biotechnology, USA), or catalase (1:1000; Rockland Immunochemicals, USA) diluted in PBS–BSA–Triton. They were then incubated for 2 h with biotinylated anti-rabbit IgG antibody (1:250; Vector Laboratories, USA) and then for 2 h with streptavidin conjugated to Alexa 488 (1:400; Invitrogen, France). After the immunohistochemical procedures, sections were mounted with Mowiol and visualized under a Zeiss microscope (Axiophot).

Analysis of ROS production

Because limitations to the current analytical approaches to detect ROS have been described [34], we chose to combine two techniques for the evaluation of ROS production: in situ detection of ROS production by dihydroethidine and cellular redox status with carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-H_2DCFDA).

In situ detection of ROS production

Dihydroethidine (DHE; 50 mg/ml in dimethyl sulfoxide; Invitrogen) was diluted 1:50 in 0.05 M PBS at 37 °C.

Mice were given an ip injection of 300 μl DHE 48 and 24 h before being killed [35]. Mice were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Frozen brain sections (20 μm) were observed with a Zeiss microscope.

To detect ROS production in hypothalamic slices, slices were incubated for 45 min in the dark in 2 μM DHE diluted in aCSF [36]. After treatment, the slices were fixed, frozen, and sectioned as described above and fluorescence was analyzed.

Evaluation of the redox status

Redox status was assessed with the fluorescent redox-sensitive dye carboxy-H_2DCFDA (50 mM in dimethyl sulfoxide; Invitrogen) [10].

Wild-type, Tg8, and α-MPT-treated Tg8 mice were anesthetized (pentobarbital, 25 mg/kg ip) and the brains removed. The SON was punched out of thick frontal sections (400 μm thick) of the hypothalamus area. For hypothalamic slices, the SON was punched out at the end of the pharmacological treatment.

Punches were homogenized in 50 μl of 150 mM KCl, 20 mM Tris, 0.5 mM EDTA, 1 mM $MgCl_2$, 5 mM glucose, and 0.5 mM octanoic acid,