Angiotensin II (AngII) induces the expression of suppressor of cytokine signaling (SOCS)-3 in rat hypothalamus – a mechanism for desensitization of AngII signaling

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Abstract

Angiotensin II exerts a potent dypsogenic stimulus on the hypothalamus, which contributes to its centrally mediated participation in the control of water balance and blood pressure. Repetitive intracerebroventricular (i.c.v.) injections of angiotensin II lead to a loss of effect characterized as physiological desensitization to the peptide’s action. In the present study, we demonstrate that angiotensin II induces the expression of suppressor of cytokine signaling (SOCS)-3 via angiotensin receptor 1 (AT1) and JAK-2, mostly located at the median preoptic lateral and antero-dorsal preoptic nuclei. SOCS-3 produces an inhibitory effect upon the signal transduction pathways of several cytokines and hormones that employ members of the JAK/STAT families as intermediaries. The partial inhibition of SOCS-3 translation by antisense oligonucleotide was sufficient to significantly reduce the refractoriness of repetitive i.c.v. angiotensin II injections, as evaluated by water ingestion. Thus, by acting through AT1 on the hypothalamus, angiotensin II induces the expression of SOCS-3 which, in turn, blocks further activation of the pathway and consequently leads to desensitization to angiotensin II stimuli concerning its dypsogenic effect.


Introduction

Angiotensin II (AngII) is an octapeptide product of the cleavage of angiotensin I by angiotensin converting enzyme. It acts by specifically binding and activating at least two well characterized transmembrane G-protein-coupled receptors belonging to the seven spanning transmembrane receptor family, angiotensin receptor 1 (AT1) and angiotensin receptor 2 (AT2) (Inagami et al. 1999). At least two different intracellular pathways participate in the transduction of the AngII signal in target cells. First, the activation of Gq protein leads to inositol 1,4,5-triphosphate generation and a subsequent rise in cytosolic free calcium, which modulates cell contractility, vesicular trafficking, gene transcription and mitogenesis (Alexander et al. 1985, Sadoshima et al. 1995, Walsh et al. 1995). Secondly, the activation of JAK-2 (Marrero et al. 1995), an intracellular kinase commonly engaged by receptors belonging to class I and class II cytokine receptor families (e.g. interleukin (IL)-2, IL-4, IL-6, IL-12, growth hormone, prolactin, leptin, interferon (INF)-α, INF-β, INF-γ), which rapidly direct the signal towards the nucleus through the signal-transducer-and-activator-of-transcription (STAT) proteins (Darnell et al. 1994, Marrero et al. 1995, Schindler & Darnell 1995).

AngII plays an important role in the control of water and salt balance. Most of this action occurs through the induction of a brain response. Several areas of the central nervous system (CNS) express receptors for AngII. Cell bodies at the subfornical organ (SFO), hypothalamic paraventricular nucleus (PVN), medial preoptic lateral nucleus (MPOL), antero-dorsal preoptic nucleus (ADP) and organum vasculosum of the lamina terminalis (OVLT) express high concentrations of AT1 (Bunnemann et al. 1992), which responds rapidly to an AngII stimulus (Xu & Xinghong 1999). Neurons from supra optic nucleus (SON) also express AT1, but at lower concentrations than SFO, PVN, MPOL and OVLT. Most studies conclude that centrally administered AngII induces thirst by activating AT1 receptors in neurons of the preoptic (MPOL and ADP) and OVLT areas (Fitzsimons 1998).

In recent years, a family of proteins named SOCS (suppressor of cytokine signaling) have been characterized and shown to participate in the medium- and long-term control of ligand-induced receptor activation (Masuhara et al. 1997, Hilton et al. 1998, Yasukawa et al. 2000).
SOCS proteins are under the transcriptional control of members of the STAT family (Auernhammer et al. 1999, Krebs & Hilton 2001). Once induced, SOCS proteins target receptor/JAK complexes utilizing a central SH2 domain and a C-terminal SOCS-box domain (Endo et al. 1997, Suzuki et al. 1998, Zhang et al. 1999). In IL-6 signaling, for example, SOCS-3 transcripts are detected as early as 30 min following IL-6 treatment of cultured cells (Schmitz et al. 2000). The translated SOCS-3 then migrates to the cytoplasm, where it interacts with Tyr759 in the IL-6 receptor subunit gp130, therefore participating in the inhibition of further activation of the IL-6/JAK-2/STAT signaling pathway (Schmitz et al. 2000). In hypothalamic leptin signaling the induction of SOCS-3 seems to occur later and to generate refractoriness that lasts for approximately 20–24 h (Bjorbaek et al. 1998, Emilson et al. 1999). In a recent study, it was demonstrated that AngII is capable of inducing SOCS-3 expression in heart and consequently modulates AngII–c-Jun expression (Calegari et al. 2003). Thus, in addition to the already known intracellular systems that participate in the control of hormones, growth factors or cytokines signaling, which include tyrosine phosphatases, serine–threonine phosphatases, protein kinase C (PKC) and protein–inhibitor–of–activated–signal–transduction–and–activator–of–transcription (PIASs), the SOCS family seems to exert control of hormones, growth factors or cytokines signaling, for example, SOCS-3 transcripts are detected as early as 30 min following IL-6 treatment of cultured cells (Schmitz et al. 2000).

**Materials and Methods**

**Antibodies and chemicals**

SDS/PAGE and immunoblotting reagents were obtained from Bio–Rad (Richmond, CA, USA). Hepes, PMSF, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, AngII and BSA (fraction V) were from Sigma (St Louis, MO, USA). Losartan was from Merck Pharmaceutical Co. (Wilmington, DE, USA). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden). Protein A and nitrocellulose membranes were from Amersham Corp. (Aylesbury, Bucks, UK). Antibodies against AT1 (rabbit polyclonal, sc-579, for immunohistochemistry), JAK-2 (rabbit polyclonal, sc-7229, for immunoprecipitation), phospho[Tyr^{201}]-STAT-1 (goat polyclonal, sc-7988, for immunoblot), STAT-3 (rabbit polyclonal, sc-482, for immunoprecipitation), SOCS-3 (rabbit polyclonal, sc-9023, for immunoprecipitation and immunohistochemistry; goat polyclonal, sc-7009, for immunohistochemistry and for immunoblot) and phosphotyrosine (mouse monoclonal, sc-508, for immunoblot) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies and conjugated complexes utilized in immunohistochemistry were from Vector Laboratories, Inc. (Burlingame, CA, USA). Sodium amobarbital was from Eli Lilly (Indianapolis, IN, USA). Chemicals employed in RT-PCR experiments, primers for SOCS-3 (sense 5'-ACC TCT CTC CTC CAA CG-3' and antisense 5'-TGC TGG GCT AAC TGG-3'), β-actin (sense 5'-TTT GGG AGG GTG AGG GAC TTC-3' and antisense 5'-TGA GCA GTA CTC TGT GTG G-3') and phosphorothioate modified oligodeoxynucleotides for SOCS-3 (sense, 5'-CAT GGT CAC CCA CAG-C3' and antisense 5'-CTG TGG GTG ACC ATG-3'), were synthesized by Life Technology (Life Technologies, Gibco BRL, Gaithersburg, MD, USA).

**Animals and surgical procedures**

Male Wistar–Hannover rats (12 weeks old, 250–280 g) from the University of Campinas Animal Breeding Center had an i.c.v. cannula chronically inserted and were kept under controlled temperature (25°C) and lighting conditions (0700 h–1900 h) in individual metabolic cages, with free access to tap water and standard laboratory rodent chow. The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the study. Briefly, the animals were anesthetized with sodium amobarbital (15 mg/kg body wt, i.p.) and, after loss of corneal and pedal reflexes, were positioned on a Stoelting stereotaxic apparatus. A 23-gauge guide stainless steel cannula with indwelling 30-gauge obturator was stereotaxically implanted into the lateral cerebral ventricle using previously reported techniques and pre-established coordinates: anteroposterior, 0–2 mm from bregma, lateral 1.5 mm and vertical 4.2 mm (Michelotto et al. 2002). Rats were allowed one-week recovery before testing for cannula patency and position. Cannulas were considered patent and correctly positioned if a dypsogenic response was elicited after AngII injection (Johnson & Epstein 1975).

**Protocols for AngII-thirst induction evaluation**

Before experiments, the animals’ water supply was removed from the home cage. The indwelling obturator was replaced by a 30-gauge stainless steel injector at the end of a PE-10 tubing connected to a 10 µl Hamilton syringe entirely loaded with test solution. Immediately thereafter, AngII (at the doses stated in Fig. 1a for dose–response evaluation, or 300 pmol in 2 µl saline for the remaining experiments) or a similar volume of saline (vehicle) was injected into the lateral ventricle. To examine the thirst–induced effect after i.c.v. AngII administration, rats were randomly assigned to one of the
following protocol groups: (a) single dose saline injection: rats received 2 µl saline and the volume of water drunk was measured during the next 30 min; (b) single dose AngII injection: rats received 2 µl AngII and the volume of water drunk was measured during the next 30 min; (c) one dose AngII injection preceded by saline injection: rats received 2 µl saline and after 120 min received 2 µl AngII. The volume of water drunk was measured during the next 30 min after AngII; (d) one dose saline injection preceded by AngII injection: rats received 2 µl AngII and after 120 min received 2 µl saline. The volume of water drunk was measured during the next 30 min after saline; (e) two doses AngII injection: rats received a first dose of 2 µl AngII and after 120 min received a second dose of 2 µl AngII. The volume of water drunk was measured during the next 30 min after the second dose of AngII.

The experimental protocols designed to evaluate the AngII-thirst induction were performed in rats treated or not with 4 nmol (i.c.v. in 2 µl TE (10 mM Tris/Cl, 1 mM EDTA, pH 7.6) buffer) sense or antisense SOCS-3 oligonucleotides, according to the protocols described in the preceding section, were anesthetized and subjected to craniotomy. Hypothalami were obtained from the A260:A280 nm ratio. After 24 h, isolated total RNA was rendered genomic DNA-free by digestion with RNase-free DNase (RQ1; Promega, Madison, WI, USA).

**Tissue extraction, immunoprecipitation and immunoblotting**

Tissue extraction, immunoprecipitation and immunoblotting were performed as previously described (Carvalheira et al. 2001). Briefly, rats previously i.c.v. cannulated and treated with AngII, losartan (3·0 nmol in 2 µl saline), losartan followed (after 30 min) by AngII or saline, with or without pretreatment with phosphorothioate modified oligonucleotides, according to the protocols described in the preceding section, were anesthetized and subjected to craniotomy. Hypothalami were obtained and homogenized in freshly prepared ice-cold buffer (1% Triton X-100, 100 mM Tris, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0·01 mg aprotinin/ml). Insoluble material was removed by centrifugation (10 000 g) for 25 min at 4 °C. Aliquots of the resulting supernatants containing 2·0 mg total protein (protein determination by the Bradford method) (Bradford 1976) were used for immunoprecipitation with specific antibodies at 4 °C overnight, followed by addition of Protein A Sepharose 6 MB for 2 h. The pellets were washed three times in ice-cold buffer (0·5% Triton X-100, 100 mM Tris, pH 7·4, 10 mM EDTA and 2 mM sodium vanadate), and then resuspended in Laemmlı sample buffer (Laemmli 1970) and boiled for 5 min prior to SDS-PAGE in a miniature slab gel apparatus (Bio Rad). Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant). The nitrocellulose transfers were probed with specific antibodies. The blots were subsequently incubated with [125I] protein A. Results were visualized by autoradiography using pre-flashed Kodak XAR film. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software, ScionCorp, Frederick, MD, USA). For immunoblot of total protein extracts 0·2 mg total protein was suspended in Laemmli sample buffer, boiled for 5 min and loaded onto the electrophoresis gel. SDS-PAGE, electrotransfer and blot followed the same steps as described above for immunoprecipitation. To ensure equal loading, membranes were stained with the Coomasie Brilliant Blue dye before blotting. Only homogeneously stained membranes were employed in the study.

**RNA isolation**

Hypothalami were excised and rapidly frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (Life Technologies), according to the recommendations of the manufacturer. Total RNA was quantified by spectrophotometry at A260 nm and integrity was determined from the A260:A280 nm ratio. After 24 h, isolated total RNA was rendered genomic DNA-free by digestion with RNase-free DNase (RQ1; Promega, Madison, WI, USA).

**Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)**

RT-PCR was employed to determine mRNA expression for SOCS-3 using β-actin as an internal standard. Seven micrograms total RNA from each separate hypothalamus were reverse-transcribed with SuperScript enzyme reverse transcriptase (200 U/µl) using oligo (dT) (50 mM) in a 30 µl reaction (5 × RT buffer, 10 mM dNTP, and 40 U/µl RNase free inhibitor). The reverse transcriptions were performed as follows: 50 min at 42 °C and 15 min at 70 °C. Following reverse transcription, 0·75 µl of the RT product was used in each PCR, in a final volume of 50 µl (10 × PCR buffer, 1·0 mM dNTP, 50 mM MgCl₂, Taq polymerase and sense and antisense primers for SOCS-3 and β-actin). mRNA expression was determined by PCR using the primers as stated in the Antibodies and chemicals subsection, and amplified a 251 bp DNA fragment of SOCS-3, and a 489 bp DNA fragment of β-actin. Triplicates of PCR were carried out as follows: initial incubation at 94 °C for 5 min, denaturation at 94 °C for 1 min followed by annealing at 52 °C for 50 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. A cycle titration between 20 and 40 cycles of RT-PCR products revealed that 23 cycles for β-actin and 30 cycles for SOCS-3 were within the logarithmic phase of amplification. Therefore, these PCR conditions were used for subsequent experiments. All PCR experiments included one control tube with no RT step. PCR-amplified products were resolved in 2% Tris/acetate acid/EDTA at 40 °C.
Phosphate-buffered saline for 1 h at 37 °C, followed by overnight incubation with the primary antibody (rabbit anti-SOCS polyclonal – 1:20 dilution) in 1% BSA in phosphate-buffered saline for 30 min to quench the endogenous peroxidase activity. The sections were pretreated in a microwave oven in sodium citrate buffer (pH 7-4) for 10 min. After being washed in phosphate-buffered saline, the sections were blocked with 3% nonfat dry milk in phosphate-buffered saline, pH 7-4, for 10 min at room temperature, the sections were incubated in 1% H₂O₂ in phosphate-buffered saline for 30 min to quench the endogenous peroxidase activity. The sections were then incubated in 1% H₂O₂ in phosphate-buffered saline, pH 7-4, for 10 min at room temperature, followed by overnight incubation with the primary antibody (rabbit anti-SOCS polyclonal – 1:20 dilution) in 1% BSA in phosphate-buffered saline at 4 °C in a moisture chamber.

After incubation with the primary antibody, sections were washed and incubated with a specific biotinylated antirabbit secondary antibody (Vector Laboratories; 1:150 dilution) for 2 h at room temperature, followed by incubation with streptavidin reagent (containing avidin-conjugated peroxidase) and color reaction using the DAB substrate kit (Vector Laboratories) according to the recommendation of the manufacturer. After the color reaction, sections were counterstained with Harris hematoxylin, dehydrated through an ethanol series into xylene and mounted using Entelan mounting media (Microscopy, Germany). Secondary antibody specificity was tested in a series of positive and negative control measurements. In the absence of primary antibodies, application of secondary antibodies (negative controls) failed to produce any significant staining. The images were obtained using an optical microscope (Leica, Wetzlar, Germany) and acquired by a Focus Imagecoder Plus system.

For double immunofluorescence staining, 5 µm sections of 4% paraformaldehyde fixed hypothalami were incubated for 30 min at room temperature with 2% preimmune rabbit serum followed by 30 min at room temperature with pre-immune goat serum. Sections were then exposed during 12 h at 4 °C to goat anti-SOCS-3 antibody (1:100) followed by 2 h at room temperature to rabbit anti-AT1 antibody. For signal detection, FITC-conjugated anti-rabbit and rhodamine-conjugated antigood secondary antibodies were employed (Araujo et al. 2002). Image acquisition was performed using a Zeiss LSM 510 laser scanning confocal microscope.

Data presentation and statistical analysis

All numerical results are expressed as the mean ± S.E.M. of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software (ScionCorp). The Tukey–Kramer test (ANOVA) was used for statistical analysis. The level of significance was set at P<0.05.

Results

AngII-induced water consumption response and refractoriness

The induction of water consumption is one of the most reproducible physiological effects of centrally administered AngII (Fitzsimons 1998). Following an acute i.c.v. injection of a dose as low as 50 ng AngII, a rapid drinking response is elicited. However, if in a short space of time, a second similar dose of AngII is i.c.v. injected in the same animal a drinking response of much lower magnitude is observed, a phenomenon produced by desensitization to the AngII signal. To evaluate the possible participation of SOCS-3 in the control of hypothalamic AngII signaling, a protocol of thirst induction and desensitization to the AngII signal was developed. Initially, in a dose–response experiment (Fig. 1a), a dose of 300 pmol AngII was elected as the optimal for the rest of the study. A single dose of saline injected i.c.v. (protocol a in Materials and Methods, Protocols for AngII-thirst induction evaluation) led to a mean volume of 1·9 ml (S.E.M.=0·4 ml, n=5) water consumption over 30 min (Fig. 1a), while a single dose of AngII (300 pmol) (protocol b) promoted the consumption of 9·9 ml (S.E.M.=0·7 ml, n=5) water in 30 min (P<0.05) (Fig. 1a). When two subsequent doses of AngII were i.c.v. injected (protocol c), a volume of 10·4 ml (S.E.M.=0·4 ml, n=5) was consumed after the first dose, while a volume of only 1·7 ml (S.E.M.=0·2 ml, n=5) was consumed after the second dose (P<0.05) (Fig. 1b). Previous or subsequent injection of saline (protocols c and d) promoted no significant changes in the volume of water consumed following a single dose of AngII (not shown). Finally, the intragastric administration of 10·0 ml water (which approximately matches the volume drunk after a single AngII dose) just before a single dose of i.c.v. AngII did not significantly change the volume of water consumed after treatment with AngII (Fig. 1c). Thus, the significant reduction of water volume drunk after the second dose of AngII is not due to gastric distension or
liquid-induced thirst inhibition, but is due to hypothalamic desensitization to AngII signaling.

AngII-induced SOCS-3 expression

Since AngII activates the JAK-2/STAT signaling pathway, we decided to investigate the capacity of i.c.v.-injected AngII to induce the expression of SOCS-3 in rat hypothalamus. Two methods were employed for this study. In the first series of experiments, saline or AngII were i.c.v. injected as a single dose, and after 0, 15, 30 or 60 min the animals were killed and hypothalami were obtained for RNA extraction. RT-PCR demonstrated that no change in β-actin expression was detected after the challenge with AngII. Conversely, the treatment with AngII led to an increase of up to 1.8-fold (P, 0.05) in SOCS-3 mRNA expression after 60 min. The characteristic pattern of SOCS-3 expression was indicative of transcriptional modulation, since a stepwise increase was evident during the time-course evaluated (Fig. 2a). In a second approach, rats were i.c.v. injected with saline or 300 pmol AngII and after 0, 15, 30, 60 and 120 min the hypothalami were obtained and homogenized in protein extraction buffer. Protein extracts containing 2.0 mg total protein were employed in immunoprecipitation experiments utilizing anti-JAK-2 antibodies. Immunocomplexes were captured by Protein A-Sepharose and resolved in SDS-PAGE. Nitrocellulose transfers were blotted with anti-SOCS-3 antibodies. As shown in Fig. 2b, AngII induced an increase of up to 1.8-fold (P, 0.05) in the SOCS-3/JAK-2 association beginning at 30 min and remaining steady until 120 min. The evaluation of SOCS-3 in hypothalami extracts by immunoblot of total extracts resulted in no detectable bands even after AngII stimulus (not shown). We suspect that the total amount of SOCS-3 in hypothalamic extracts is below the sensitivity of the method, and only after partial purification, as in immunoprecipitation/association experiments (as detected in JAK-2 immunoprecipitates) is it possible to detect and measure SOCS-3 in hypothalamus. To further evaluate the physiological significance of this phenomenon, a dose–response experiment was undertaken and a significant induction of AngII upon SOCS-3 expression in rat hypothalamus was detected with 300 pmol AngII (Fig. 2c).

In other signaling systems known to induce SOCS-3 expression, proteins of the STAT family were shown to participate in the induction of SOCS-3 gene transcription. By activating JAK–2, AngII promotes the phosphorylation of members of the STAT family. To test the integrity of the JAK–2/STAT signaling pathway in hypothalamus and to investigate the ability of AngII to promote the activation of elements of this signal transduction cascade, AngII-induced tyrosine phosphorylation of JAK-2, STAT-1 and STAT-3 were evaluated in hypothalamic protein extracts obtained from rats i.c.v. injected with

Figure 1 Effect of i.c.v. injection of AngII upon drinking behavior of rats. (a) Rats were i.c.v. cannulated and received 2.0 μl saline or 2.0 μl of a solution containing AngII (amounts stated in the figure). The volume of spontaneous water consumption was measured over the next 30 min. (b) Rats were i.c.v. cannulated and two subsequent i.c.v. doses of AngII (300 pmol) were injected with an interval of 120 min. Spontaneous water consumption (DW) was measured 30 min after each dose. (c) Rats were i.c.v. cannulated and treated either with a single i.c.v. dose of AngII (300 pmol) (AngII) or previously submitted to gastric tubing and received an infusion of 10 ml water into the stomach immediately before receiving an injection of a single i.c.v. dose of AngII (300 pmol) (GT+AngII). In both groups, spontaneous water consumption was measured over the next 30 min. In all experiments n=5; *P<0.05 vs saline-treated in (a) and vs. 1st i.c.v. AngII in (b).
Figure 2 Effects of AngII upon SOCS-3 expression and the activation of JAK-2/STAT-1 and -3 signal transduction in hypothalamus of rats. In all experiments, rats were i.c.v. cannulated and treated with 2 µl saline or 300 pmol AngII (or with the dose stated in c). After the times stated in the figure (30 min in c), hypothalami were obtained and RNA (a) or total protein (b–f) were extracted according to the protocols described in Materials and Methods. (a) SOCS-3 mRNA expression was evaluated by RT-PCR in parallel with the expression of β-actin. (b–d) Protein extracts were submitted to immunoprecipitation (IP) with anti-JAK-2 antibodies. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-SOCS-3 (b, c) or anti-phosphotyrosine (pY) (d) antibodies. (e) Total protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-phospho-STAT-1 (p-STAT-1) antibody. (f) Protein extracts were submitted to immunoprecipitation with anti-STAT-3 antibodies. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-phosphotyrosine (pY) antibody. In all experiments n = 5; *P < 0.05 vs rats not treated with AngII.
AngII employs the AT1 receptor to control water ingestion and SOCS-3 expression

To evaluate the participation of the AT1 receptor in AngII-induced water consumption and SOCS-3 expression, rats were i.c.v. cannulated and submitted to an AngII dose–response protocol for evaluation of water consumption over 30 min, in the presence or absence of losartan (3-0 nmol in 2-0 μl saline). As depicted in Fig. 3a, losartan blocked AngII-induced water consumption when the doses of AngII were 30 and 300 pmol. At higher doses some residual effect of AngII could be detected. Moreover, the pre-treatment of i.c.v. cannulated rats with losartan abolished AngII-induced JAK-2 tyrosine phosphorylation and JAK-2/SOCS-3 association (Fig. 3b and c respectively).

Immunohistochemical localization of SOCS-3 expression in hypothalamus

AngII-induced modulation of drinking behavior is mediated by AT1 receptors of the anterior hypothalamus (Xu & Xinghong 1999). To investigate the anatomical distribution of AngII-induced SOCS-3, we performed immunohistochemical evaluations of histological sections obtained from anterior, medium and posterior hypothalamus of saline- or AngII-treated rats. Specimens were obtained 2 h after i.c.v. treatment. In the hypothalamus of saline-treated rats, SOCS-3 expression was absent or was observed as a faint stain in some areas of the posterior hypothalamus in the vicinity of the magnocellular preoptic nucleus. Following AngII administration, an impressive expression of SOCS-3 was evident in neurons of the MPOL and ADP (Fig. 4c). Double immunofluorescence studies evaluated by confocal microscopy revealed the co-localization of AT1 and SOCS-3 in ADP (Fig. 4d) and MPOL (Fig. 4e).

AngII-induced thirst response desensitization is abrogated by SOCS-3 antisense oligonucleotide treatment

Since SOCS-3 is a well-known controller of cytokine and hormone signaling, we decided to test the hypothesis that AngII-induced SOCS-3 expression participates in the mechanisms of hormone desensitization in the hypothalamus by blocking SOCS-3 expression utilizing a SOCS-3 antisense phosphorothioate modified oligonucleotide. Based on the Rattus norvegicus SOCS-3 mRNA sequence (accession no. AF075383 at NCBI Entrez Nucleotide), three different sequences were designed and tested by i.c.v. injection of 4 nmol antisense or respective sense oligonucleotide, and evaluated for its...
property of blocking SOCS-3 synthesis by measuring SOCS-3 associated to JAK-2 in immunoprecipitates of hypothalamic protein extracts. The sequence 5'-CTG TGG GTG ACC ATG-3' was capable of reducing 80% (P<0.05) basal or AngII-stimulated SOCS-3 association to JAK-2 and was utilized in all experiments in parallel with its respective sense sequence as control (Fig. 5a). All the protocols stated in Materials and Methods (Protocols for AngII-thirst induction evaluation) were repeated in the presence of sense or antisense SOCS-3 oligonucleotide (i.c.v. injected 20 min before first dose of saline or AngII) and measurements of water drinking volume were performed. As depicted in Fig. 5 (b and c), pretreatment with SOCS-3 antisense but not with sense oligonucleotide significantly reversed the desensitization of AngII as an inducer of water drinking.

Discussion

Cellular regulation of signaling inputs must be coordinated in such a way that over exposure of a given tissue or cell to an extra-cellular signal does not lead to a progressive and uncontrolled response. Several systems participate in the control of signal transduction. Thus, tyrosine phosphatases actively participate in the regulation of signal transduction, through tyrosine kinase receptors, by promoting receptor or substrate tyrosine dephosphorylation. Serine and threonine kinases or phosphatases may act to enhance and suppress signal transduction (Cross et al. 2000, Summers et al. 2000), whilst phosphoinositide phosphatases such as SHIP1 and SHIP2 modulate the signaling cascades that depend on phosphorylated membrane phosphoinositide (Osborne et al. 1996, Clement et al. 2001, Bertelli et al. 2003). In contrast to enzymatic interference in signaling pathways, such as that observed with kinases or phosphatases, some systems may be regulated by physical blockade of the signal transducers’ functional sites. This seems to be the case for PIASs and members of the SOCS family. PIASs embrace a family of proteins that modulate signal transduction through members of the STAT family by binding to the signal transducers and to consensus DNA binding sites, acting either as a coactivator or as a blocker (Greenhalgh & Hilton 2001). The SOCS protein family is composed of eight members (CIS and SOCS-1 to SOCS-7) (Yoshimura et al. 1995, Endo et al. 1997, Naka et al. 1997, Starr et al. 1997), all of them possessing a common structure that displays a variable N-terminal region, a central SH2 domain, and a C-terminal tail, named the SOCS box motif (Hilton et al. 1998). The SH2 domain participates in protein–protein interaction, while the SOCS box motif assures protection for SOCS proteins against ubiquitination and proteasomal driven degradation, thus extending the half life of the protein complex and allowing for longer blockade (Zhang et al. 1999). SOCS proteins act by targeting members of the JAK family and interfering with downstream steps of their signaling cascade. Several cytokines and some hormones that employ JAK/STAT for transducing their signal are known to induce SOCS expression and association with JAK proteins. Previously, only cytokines or hormones that act through receptors of the cytokine receptor or tyrosine kinase receptor families have been shown to induce SOCS activation (e.g. IL-6, IL-11, leukemia inhibitory factor

Figure 4 Immunohistochemical evaluation of AngII-induced SOCS-3 expression in anterior hypothalamus. Rats were i.c.v. cannulated and received 2 µl saline (insets in d–e) or the same volume of a solution containing 300 pmol AngII (c–e). After 2 h, the rats were perfused with 4% paraformaldehyde in 0.02 M phosphate-buffered saline (pH 7.4). Fixed hypothalami were embedded in paraffin, sectioned, and submitted to a staining protocol as stated in Materials and Methods. According to immunoperoxidase staining, AngII induced the expression of SOCS-3 in median preoptic lateral nucleus (MPOL) and anterodorsal preoptic nucleus (ADP) (c). By double-staining immunofluorescence evaluated by confocal microscopy (d, e), SOCS-3 is shown to co-localize with AT1 receptor in ADP (d) and MPOL (e). The coordinates of the section are depicted in (a) and (b). Third ventricle (3v), optic chiasm (ox), median preoptic area (MPA), periventricular nucleus (Pe), anterointerventricular periventricular nucleus (AVPe), striatal part of preoptic area (StA), VMPO, ventromedial posterior nucleus.
As in the hypothalamus, in rat heart the expression of SOCS-3 is induced by AngII through AT1, and the blockade of SOCS-3 expression by antisense oligonucleotide treatment restores the capacity of AngII to induce c-jun expression after a first stimulus (Calegari et al. 2003). Therefore, a reversal of AngII desensitization was evidenced at the molecular level. In the present report, the participation of SOCS-3 in AngII desensitization was investigated in another tissue known to respond promptly to an AngII stimulus, and for that we evaluated a well-known physiological phenomenon triggered by AngII, the induction of water intake (Fitzsimons 1998).

Refractoriness to repetitive i.c.v. AngII injections is a well-documented phenomenon (Sakuta et al. 1991, Thomas et al. 1995, 1996), which is not a consequence of reduced osmolarity due to high liquid ingestion, since the offering of osmotically balanced liquids to experimental animals does not influence the drinking behavior pattern. In the present experiments, repetitive i.c.v. injections of 300 pmol AngII provoked a reduction of more than 80% in the volume of ingested water following the second dose of AngII. In rats primed with a similar volume of i.c.v. saline and treated (through a transoral gastric tube) with the same mean volume of water as detected in the animals that received AngII, a normal response to i.c.v. AngII injection was detected. Thus, either the offer of osmotically balanced liquid to animals primed with i.c.v. AngII or the gastric infusion of water in i.c.v. saline-primed rats provoked no change in the response to centrally administered AngII. Therefore, it seems that refractoriness to AngII in the hypothalamus could be mediated by an AngII inducible factor that may block further signaling through AT1.

**Figure 5** Effect of SOCS-3 partial blockade upon AngII-elicited drinking behavior of rats. Rats were i.c.v. cannulated and received either SOCS-3 antisense (a and b) or SOCS-3 sense (a and c) phosphorothioate modified oligonucleotides (4.0 nmol in 2.0 μl solution). (a) A dose of 300 pmol AngII (2.0 μl solution) was i.c.v. injected and after 30 min the amount of SOCS-3 associated to JAK-2 in the hypothalamus was determined by immunoprecipitation (IP) of total protein extracts utilizing anti-JAK-2 antibody. The immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-SOCS-3 antibody. (b and c) AngII (300 pmol) was injected and water consumption was measured over the next 30 min (1st i.c.v. AngII) (DW(A)). Ninety minutes after the conclusion of the water intake measurement, a second dose of 300 pmol AngII was i.c.v. injected and the volume of water ingested was measured over the next 30 min (2nd i.c.v. AngII) (DW(B)). In all experiments n = 5; in c *P < 0.05 vs 1st i.c.v. AngII.
Distinct mechanisms have been shown to participate in AngII signal desensitization. Recycling of the receptor, leading to a reduction in the extracellular presence of the hormone sensing apparatus, was one of the first mechanisms to be studied (Sasamura et al. 1994). AngII-induced internalization of AT1 was later demonstrated and shown to be dependent on its uncoupling from G-protein (Boulay et al. 1994, Tang et al. 1995). Also, the participation of PKC-induced serine phosphorylation of AT1 was shown to participate in receptor desensitization at low physiological levels of AngII (Sterne-Marr & Benovic 1995, Balnforth et al. 1997). Finally, in a recent study, the induction of the early inducible genes, c-fos and c-jun, was demonstrated to be implicated in the control of expression of transcription factors that ultimately mediate the desensitization to the AngII signal (Moellenhoff et al. 2001). In spite of the efforts to characterize the desensitization of AngII signaling, none of the mechanisms so far identified seem to act independently and, as it now stands, desensitization seems to be the result of different mechanisms that participate in concert to modulate the response to AngII.

In the present experimental model, i.c.v. administration of AngII led to an increase in the level of SOCS-3 mRNA, and in the JAK-2/SOCS-3 association. Previous i.c.v. administration of the AT1 blocker, losartan, significantly prevented the JAK-2/SOCS-3 association, a fact that, taken together with the pattern of histological distribution of SOCS-3 and AT1, strongly suggests that the effect of AngII upon SOCS-3 expression is mediated by AT1. In saline-treated rats, only faint and occasional staining of SOCS-3 was detected in some areas of the hypothalamus. Following AngII stimulus, an impressive change in the staining pattern was observed with a strong and compact labeling of MPOL and ADP neurons. According to previous studies (Simonnet et al. 1979) neuron bodies of the anterior hypothalamic preoptic area together with OVLT neurons may act as primary sites for AngII action in the control of water balance and induction of drinking behavior. Thus, the coincident expression of SOCS-3 in neurons of some of these areas reinforces its possible participation in important steps of AngII signaling in the hypothalamus. Finally, by blocking SOCS-3 expression by i.c.v. injection of an antisense oligonucleotide specific for SOCS-3, a partial (but significant) reversal of AngII refractoriness was obtained.

In conclusion, the present data provide strong evidence of the induction of an intracellular signal transduction controller, classically involved in cytokine and related hormone signaling by AngII. Together with our recent report evaluating AngII-induced SOCS-3 expression in the heart, this is the first evidence of the participation of a member of the SOCS family in the modulation of a signal transduced by a receptor belonging to the G-protein-coupled receptor family. Finally, since abnormal response to AngII may play an important role in the pathogenesis of cardiovascular and metabolic diseases, the identification of novel mechanisms that participate in the control of its signal transduction may offer new targets for therapeutic approaches in the control of hypertension, cardiac and vascular wall hypertrophy, among others.

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