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Enteral arginine modulates inhibition of AP-1/c-Jun by SP600125 in the postischemic gut

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Abstract

We previously demonstrated that enteral arginine increased c-Jun/activator protein-1 (AP-1) DNA-binding activity and iNOS expression in a rodent model of mesenteric ischemia/reperfusion (I/R). The objective of this study was to specifically investigate the role of AP-1 in arginine's deleterious effect on the postischemic gut. We hypothesized that AP-1 inhibition would mitigate the effects of arginine. Using a rodent model of mesenteric I/R we demonstrated that gut neutrophil infiltration, activity of c-Jun/AP-1, as well as iNOS expression were increased by I/R and further increased by arginine while lessened by inhibition of c-Jun using the pharmacologic c-Jun N-terminal kinase inhibitor, SP600125. Similar results were demonstrated using a cell culture model of oxidant stress in IEC-6 cells. Importantly, effects of SP600125 were comparable to those of c-Jun silencing. Lastly, the specific iNOS inhibitor, 1400W, had no effect on either AP-1 or c-Jun. In conclusion, SP600125 attenuated the activity of c-Jun/AP-1, iNOS expression, and neutrophil infiltration induced by arginine following mesenteric I/R. Our data suggest that AP-1 inhibition mitigates the injurious inflammatory effects of arginine in the postischemic gut. Further investigation into the pathologic role of enteral arginine in the postischemic gut is warranted.

Keywords

Arginine; SP600125; Activator protein-1; Mesenteric ischemia/reperfusion; Inflammation; Inducible nitric oxide synthase

Introduction

Ischemia/reperfusion (I/R) injury in the gut plays a pivotal role in dysfunctional inflammation that leads to the development of postinjury multiple organ failure. Numerous clinical studies evaluating the use of early enteral nutrition with immune enhancing nutrients have shown reduced septic morbidity and multiple organ failure in trauma patients. The immune enhancing formulas utilized in these studies were often composed of multiple

immune modulating nutrients including glutamine, arginine, omega-3- fatty acids, and/or nucleotides, making it difficult to determine which immune enhancing nutrients were beneficial or potentially harmful. Some believe that immune enhancement in states of overt inflammation is potentially harmful. Most notably, arginine supplementation is thought to amplify the systemic inflammatory response syndrome by enhancing nitric oxide production. Interestingly, we demonstrated differential induction of inflammatory mediators by enteral arginine and glutamine [1]. Glutamine increased expression of the anti-inflammatory mediator peroxisome proliferator activated receptor (PPAR) γ and was associated with gut protection. In contrast, arginine increased the proinflammatory mediator activator protein-1 (AP-1) via c-Jun and enhanced inducible nitric oxide synthase (iNOS) with associated gut injury.

AP-1 is a stress-activated DNA-binding protein composed of subunits that belong to the c-Fos and c-Jun families whose activity is regulated by the mitogen-activated protein kinases (MAPK). c-Jun N-terminal kinase (JNK), a member of the MAPK pathway, is a serine/threonine protein kinase that phosphorylates a number of transcription factors, including the c-Jun component of the AP-1 transcription factor complex. JNK is involved in inflammation, apoptosis, cell proliferation, and oncogenic transformation [2]. Specific to the gut, Mitsuyama et al. demonstrated a pathologic role for JNK in patients with inflammatory bowel disease. Inhibition of JNK by SP600125 decreased inflammatory cytokines in patients with inflammatory bowel disease and decreased intestinal inflammation in an animal model [3]. Accumulating evidence has also demonstrated that JNK was up-regulated during I/R [4–6]. JNK is a stress-activated protein kinase which can be induced by various stimuli, including reactive oxygen species, proinflammatory cytokines, osmotic and mechanical stress, and Toll-like receptor activation [7]. During I/R, tissues release proinflammatory cytokines, reactive oxygen species, and Toll-like receptor activation [8,9], in turn, activate JNK. The best characterized JNK target/substrate is phosphorylation of the amino terminus of c-Jun. Beneficial effects of JNK inhibition in I/R injury have been documented in lung [10], brain [11], and kidney [12]. However, there are no data available on JNK inhibition in gut I/R-induced inflammation. As we have previously demonstrated that arginine increases both AP-1 and iNOS after gut I/R, the objective of this study was to specifically investigate the role of AP-1 in arginine's deleterious effect on the postischemic gut. Our data suggest that AP-1 inhibition mitigates neutrophil infiltration, a hallmark of inflammation in the postischemic gut.

Materials and methods

Reagents and animals

JNK inhibitor SP600125 (JNK inhibitor II) was obtained from Calbiochem (La Jolla, CA, USA). Magnesium sulfate, arginine, protease inhibitor cocktail, and iNOS inhibitor 1400W were purchased from Sigma-Aldrich (Milwaukee, WI, USA). DMEM medium was the product of Invitrogen Corp. (Carlsbad, CA, USA). Antibodies against phospho-c-Jun (Ser63) II, c-Jun, and β -actin were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody against iNOS was obtained from BD Transduction Laboratories (San Jose, CA, USA). ECL anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey), and ECL plus Western blotting detection system were ordered from GE Healthcare (Piscataway, NJ, USA). Nuclear/Cytosol Fractionation Kit was bought from BioVision Inc. (Mountain View, CA, USA).

Animals

Animal procedures were approved by the University of Texas Houston Medical School Animal Welfare Committee. Male Sprague–Dawley rats weighing 250–300 g were obtained

and housed individually. They were kept at room temperature (25°C) with alternating 12 h light/dark cycles. Animals were fed standard rat chow and water ad libitum during a minimum acclimation period of 5 days. Each animal was fasted with free access to water overnight before laparotomy. Operative procedures were performed using sterile techniques under general anesthesia with isoflurane-inhaled anesthetic.

Mesenteric I/R model

A midline laparotomy was performed and the jejunum was identified 5 cm distal to the ligament of Treitz. An 8 cm intestinal sac was created by occluding the lumen of the bowel with 3–0 silk ligatures [1]. After creating the sac, 60 mM of L-arginine or 30 mM of magnesium sulfate (nonabsorbable, isoosmotic control) or vehicle was injected into sacs. Sixty millimolar approximates the amount of arginine in commercially available immune enhancing diets. The JNK inhibitor, 1,9 pyrazoloanthrone (SP600125), was dissolved in dimethyl sulfoxide (DMSO). SP600125 (30 mg/kg) or same volume of DMSO as that of SP600125-treated group was injected intraperitoneally into rats 1 h before ischemia [13]. Similar effects were demonstrated when SP600125 was injected directly into the enteral sac (data not shown). Mesenteric I/R was carried out by superior mesenteric artery occlusion for 1 h, based on previous studies that demonstrate consistent differences in mucosal injury between groups with no measurable mortality [1].

After removal of the clamp, the incision was closed and rats were allowed to awaken and were observed for 6 h. Sham animals underwent the identical procedure, but without placement of the clamp on the superior mesenteric artery. At the end of 6 h, animals were sacrificed under isoflurane anesthesia by exsanguination and intestinal sacs were harvested. Groups included: sham (magnesium sulfate + vehicle), sham + SP (SP600125 + magnesium sulfate), IR (I/R + magnesium sulfate + vehicle), IR + SP (I/R + SP600125 + magnesium sulfate), IR + Arg (I/R + arginine + vehicle), or IR + Arg + SP (I/R + SP600125 + arginine), six animals per group. Magnesium sulfate was used as an iso-osmotic nonabsorbable control. We have previously shown that arginine under sham conditions is not deleterious [1].

Cell culture

The normal, non-transformed rat small intestinal cell line IEC-6 (CRL-1592) was purchased from the American Type Culture Collection (Rockville, MD, USA) and routinely maintained in the presence of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2 mM L-glutamine, 5 µg/ml insulin, 1% penicillin, and 1% streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air in the incubator. All experiments were done on cells between passages 17 and 30.

Oxidant stress model

H₂O₂ was used to induce oxidative stress to mimic, in part, the stress response observed during intestinal I/R. For some experiments, cells were treated with 10 mM of arginine, 20 µM of SP600125 (dissolved in DMSO) [14], 20 µM of 1400W (dissolved in PBS) [15], or vehicle for 24 h, followed by incubation in 250 µM of hydrogen peroxide (H₂O₂) for 6 h [16]. Each group including controls was added the same volume of DMSO as that of the SP600125-treated group.

RNA silencing of c-Jun

To confirm that the effects of SP600125 were due to c-Jun repression, interference transfections were performed with the silencer siRNA Starter Kit according to the manufacturer's instructions. Pre-designed c-Jun siRNA for rat (part number: 4390815) and

negative control siRNA #1 (cat # 4611) (scrambled siRNA) were purchased from Ambion (Austin, TX, USA). The c-Jun siRNA duplex oligonucleotide sense sequence was as follows: 5'-GGCACAGCU UAAACAGAAAtt-3'.

Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic fractions in jejunum tissues from rats and IEC-6 cells were separated by Nuclear/Cytosol Fractionation Kit, according to the manufacturer's protocol. Nuclear and cytosol proteins were aliquoted and stored at -80°C for electrophoretic mobility shift assay (EMSA) and myeloperoxidase (MPO) activity assay, respectively. The protein content was measured using the Bio-Rad Protein Assay.

MPO activity

Myeloperoxidase activity was measured as an index of gut leukocyte infiltration. Ten microliters of cytoplasmic extracts from full-thickness jejunum were added to 96-well plates and incubated with 100 μl tetramethyl bezidine microwell peroxidase substrate at room temperature for 20 min. The reaction was stopped with 100 μl of 1.8 M sulfuric acid. Optical density was measured at 450 nm with an ELISA plate reader. Assays were performed in duplicate, and the results were normalized for protein content.

EMSA

The DNA-binding activity of AP-1 in nuclear extracts was determined by EMSA. The AP-1 consensus oligonucleotide 5'-CGC TTG ATG ACT CAG CCG GAA- 3' (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was end-labeled with [γ - ^{32}P] adenosine triphosphate T4 polynucleotide kinase. Ten micrograms of nuclear protein was then incubated for 20 min with gel shift binding buffer (10 mmol/l Tris [pH 7.5], 50 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 5% glycerol, and 1 μg of poly [dI-dC] and 1.0 μl of labeled probe). Cold competition was performed by adding a 200-fold molar excess of double-stranded oligonucleotide. For antibody supershift and blocking assays, the reaction mixtures were preincubated with c-Jun antibody at room temperature for 20 min before addition of the 5' ^{32}P -labeled blunt-ended double-stranded oligonucleotide. Our previous study has demonstrated that I/R and arginine increased the AP-1 component c-Jun, but not c-Fos in an I/R intestinal model [17]. Gel loading buffer was then added to the mixture and the samples were electrophoresed on the nondenaturing 5% polyacrylamide gel. Gels were dried, exposed onto films and analyzed by image-analysis software (Optimas 6.1, Cybernetics, Silver Spring, MD) and expressed in arbitrary units.

Western blot

Jejunal tissue and IEC-6 cells were lysed with RIPA buffer containing protease inhibitors. Proteins in the lysates were separated by electrophoresis on SDS-polyacrylamide gel and transferred onto a Hybond-P membrane. The membranes were blocked with 5% fat-free milk in TBS with 0.1% Tween 20 for 1 h at room temperature, and incubated overnight at 4°C with antibodies against phospho-c-Jun (Ser63) II (p-c-Jun), c-Jun, iNOS, and β -actin. The blots were then incubated for 1 h at room temperature with ECL anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey), developed with ECL plus Western blotting detection system and exposed onto films. p-c-Jun protein levels were normalized using the amount of c-Jun, and iNOS protein levels were normalized using the amount of β -actin as a standard and are expressed in arbitrary units.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Data were then analyzed by one-way analysis of variance and individual group means compared using Tukey's multiple group comparison

test. *P* values < 0.05 were considered significant. Means with different letters are significantly different.

Results

SP600125 inhibited gut inflammation

Myeloperoxidase, an enzyme located in the azurophil granules of neutrophils, is a useful indicator of neutrophil sequestration. The MPO level was increased in the IR group (8.0 ± 0.9) compared to shams (3.1 ± 0.9) and further increased by arginine in IR + Arg group (11.6 ± 1.6) (Fig. 1). SP600125 significantly decreased MPO levels in both IR + Arg + SP group (7.0 ± 0.3) and IR + SP group (5.0 ± 0.2) compared to IR + Arg and IR group. These results suggest that AP-1/c-Jun plays an important pathologic role in gut inflammation and that SP600125 provides protection.

SP600125 inhibited AP-1 and c-Jun

The JNK cascade of the mitogen-activated protein kinase (MAPK) pathway is responsible for phosphorylation and activation of c-Jun, one member of the Jun family of AP-1. The JNK inhibitor, 1,9 pyrazoloanthrone (SP600125) was utilized to examine the mechanism by which arginine increases AP-1 in the postischemic gut and demonstrated that there was a low level of AP-1 DNA-binding activity in shams (Fig. 2A). Cold competition and antibody shift assay confirmed the specificity of the probe (Fig. 2B). Enteral arginine significantly increased AP-1 in IR + arg group compared to the IR group while SP600125 reduced AP-1 in IR + Arg + SP group and further decreased in the IR + SP group (Fig. 2A). Similarly, arginine significantly increased expression of p-c-Jun in the postischemic gut in IR + arg group compared to IR group while SP600125 effectively inhibited its expression in IR + Arg + SP and IR + SP groups (Fig. 2C). In vitro results paralleled those in vivo with SP600125 demonstrating a marked reduction in AP-1 DNA-binding activity (Fig. 2D) and c-Jun expression by arginine oxidant stress conditions (Fig. 2E).

SP600125 decreased iNOS

We and others have shown that iNOS is involved in I/R-induced intestinal injury [1,18] and iNOS is known to be mediated by JNK signaling [19]. We, therefore, examined iNOS expression after inhibition of JNK by SP600125 in the postischemic gut. I/R alone increased iNOS expression which was further increased by enteral arginine while SP600125 lessened the increase of iNOS expression in both the IR and IR + arg groups (Fig. 3A). Consistent with this in vivo experiment, similar results were obtained in vitro after oxidant stress (Fig. 3B).

To further confirm that iNOS inhibition by SP600125 was due to repression of c-Jun, we silenced c-Jun and then measured iNOS expression. Figure 4 showed that the expression of phosphorylated c-Jun was silenced by siRNA (Fig. 4a) and subsequently resulted in the decrease of AP-1 activity (Fig. 4b). Western blot demonstrated that iNOS expression was decreased by c-Jun siRNA (Fig. 4a).

Inhibition of iNOS activity did not affect the activity of AP-1/c-Jun

The specific iNOS inhibitor, 1400W, is a competitive inhibitor of arginine, and has been shown to have far greater potency and selectivity in vitro and in vivo than previously described iNOS inhibitors [20]. As shown in Fig. 5, 1400W treatment changed neither the activity of c-Jun (Fig. 5A) nor AP-1 (Fig. 5B) after oxidant stress.

Discussion

We have been interested in studying the differential modulation of pro- and anti-inflammatory mediators by enteral immune enhancing nutrients in the postischemic gut [21]. Glutamine attenuated pro-inflammation by induction of the anti-inflammatory transcription factor PPAR γ while arginine induced the pro-inflammatory transcription factor AP-1 [1,22]. Interestingly, while I/R alone increased both AP-1 and NF κ B, arginine further increased AP-1 but not NF κ B [17].

To explore a new therapeutic strategy to provide protection against I/R-related inflammation, we employed the JNK inhibitor, SP600125. Results confirmed that SP600125 markedly inhibited neutrophil infiltration as measured by MPO induced by I/R and enhanced by arginine in the postischemic gut. Since neutrophil recruitment is an integral part of inflammatory disorders [23] and neutrophil infiltration is a hallmark in inflammatory diseases including inflammatory bowel diseases [24,25], we speculated that SP600125 attenuated inflammation induced by I/R and enhanced by arginine in the postischemic gut.

We have previously demonstrated that arginine increased AP-1 beyond that of I/R alone [17]. However, the role of AP-1 in inflammation induced arginine following I/R has not been studied. SP600125 has been reported to decrease not only the phosphorylation level of c-Jun protein but also AP-1 DNA-binding activity in human leukemia cell line [26]. In this study, we also demonstrated that SP600125 significantly decreased the activity of c-Jun/AP-1 and iNOS expression and consequently lessened neutrophil infiltration in the postischemic gut. Results from in vivo experiments after oxidant stress further supported the inhibition of c-Jun and AP-1 by SP600125.

iNOS has also been implicated in I/R-induced inflammation [18]. As an inflammatory mediator, iNOS acts as a cytotoxic agent and modulates immune response and inflammation and its expression is associated with inflammatory diseases. At a molecular level, the JNK pathway can mediate up-regulation of iNOS [27] and as a JNK inhibitor, SP600125 has been shown to decrease lipopolysaccharide and thermal injury-induced iNOS protein expression [28]. In contrast, others have demonstrated that blockade of AP-1 leads to the upregulation of iNOS expression in mammalian cells [29]. Thus, in this study, we attempted to investigate whether c-Jun inhibition enhanced or mitigated the increase in iNOS by arginine. Our results showed that c-Jun inhibition by SP600125 and c-Jun silencing reduced the expression of iNOS in vitro. Our findings were consistent with the evidence that SP600125 decreased peritonitis-induced pulmonary MPO activity, AP-1 DNA-binding activity, and iNOS expression in mice [30].

The JNK pathway can mediate up-regulation of iNOS expression [27], and SP600125 decreases iNOS protein expression induced by thermal injury [28]. However, there is conflicting evidence suggesting that iNOS can affect AP-1 activity. Several studies have shown that AP-1 binding activity was decreased in iNOS knockout mice compared with wild type in myocardial tissue after I/R injury [31] and in vascular smooth muscle cells after serum stimulation [32]. Results of this study demonstrated that the specific iNOS inhibitor, 1400W, failed to alter expression of c-Jun or AP-1 activity, suggesting that c-Jun/AP-1 is not a target of iNOS under our experimental conditions.

SP600125 is a reversible ATP-competitive inhibitor and its pyrazole moiety is involved in forming a critical hydrogen-bonding interaction at the ATP-binding site of JNK [33]. Although many studies have demonstrated that SP600125 inhibits JNK or AP-1 activity, [34,35] there are several reports that SP600125 may also target other proteins [33,36]. Therefore, we specifically silenced c-Jun by siRNA and results supported the finding that inhibition of c-Jun/AP-1 decreased iNOS expression.

Our finding that arginine affected AP-1 activity is supported by Leung et al. who demonstrated that arginine abrogated the effect of CCl₄-induced downregulation of AP-1 activation [37]. Precisely how arginine regulates AP-1 activity, however, is not clear. Recent reports seem to suggest that it may be an indirect mechanism via polyamine, an arginine metabolite. Bhattacharya et al. reported that depletion of polyamine prevented the activity of JNK in response to TNF- α plus cycloheximide in intestinal epithelial IEC-6 cells [38]. Polyamine depletion in the rat hepatocarcinoma cell line, FAO, impairs the activation of AP-1 and the expression of c-fos and c-Jun mRNAs induced by heat shock [39,40]. Polyamine depletion also prevents the induction of the immediate-early genes c-Jun [41]. Therefore, it is possible that arginine regulates AP-1 activity via its metabolite, polyamine.

In conclusion, SP600125 can attenuate the activity of c-Jun/AP-1 and expression of iNOS induced by arginine in the postischemic gut and in an intestinal cell culture of model of oxidant stress, a finding that can be mitigated by c-Jun inhibition. Further investigation into the pathologic role of enteral arginine in the postischemic gut is warranted.

Abbreviations

AP-1	Activator protein 1
DMSO	Dimethyl sulfoxide
EMSA	Electrophoretic mobility shift assay
H₂O₂	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
I/R	Ischemia/reperfusion
JNK	c-Jun N-terminal kinase
MPO	Myeloperoxidase
PPARγ	Peroxisome proliferator activated receptor gamma

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Fig. 1.

SP600125 (SP) decreased myeloperoxidase activity induced by ischemia/reperfusion (I/R) and arginine (Arg) in the postischemic gut. Jejunal sacs were created in rats at laparotomy and filled with 60 mM arginine or 30 mM magnesium sulfate followed by 1 h of superior mesenteric artery occlusion and 6 h of reperfusion. The JNK inhibitor, SP600125, or vehicle was injected intraperitoneally into rats 1 h prior to ischemia. Sham animals underwent the identical procedure but without placement of the clamp on the superior mesenteric artery. At the end of 6 h, intestinal sacs were harvested. Groups included: sham (magnesium sulfate + vehicle), sham + SP (SP + magnesium sulfate), IR (I/R + magnesium sulfate + vehicle), IR + SP (I/R + SP600125 + magnesium sulfate), IR + Arg (I/R + arginine + vehicle), or IR + Arg + SP (I/R + arginine + SP600125), six animals per group. Myeloperoxidase (MPO) levels in intestinal tissues were determined and results were presented as mean $\pm$ SEM ($n = 6$). Data were analyzed by one-way analysis of variance and individual group means compared using Tukey's multiple group comparison test. a vs. e $P < 0.01$, b vs. f $P < 0.05$, c vs. d $P < 0.01$, c vs. e $P < 0.05$, and e vs. f $P < 0.01$.

Fig. 2. SP600125 suppressed AP-1 DNA-binding activity and p-c-Jun expression induced by ischemia/reperfusion (I/R) and arginine (Arg) in the postischemic gut. Animal experiments were performed as described in legend of Fig. 1. In in vitro experiments, IEC-6 cells were treated with 10 mM of arginine (Arg), 20 μ M of SP600125 (SP), or vehicle for 24 h, and then were treated with H_2O_2 (250 μ M) for another 6 h. Nuclear extracts and cell lysates were prepared, and electrophoretic mobility shift assay and Western blot were performed ($n = 3$ for each group). Densitometry of bands is expressed in arbitrary densitometric units (lower panel). Values are represented as means $\pm$ SEM. **A** AP-1 activity was induced by I/R and arginine, and the increase of AP-1 activity was lessened by SP600125 in intestines: *a* vs. *e*

$P < 0.01$, b vs. f $P < 0.01$, c vs. d $P < 0.01$, c vs. e $P < 0.05$, and e vs. f $P < 0.01$. **B** Cold competition and antibody shift assays. Sample from IR + Arg group served as positive control. **C** The induction of c-Jun activity by I/R and arginine was reduced by SP600125 in intestines: a vs. e $P < 0.01$, b vs. f $P < 0.01$, c vs. d $P < 0.01$, c vs. e $P < 0.05$, and e vs. f $P < 0.01$. **D** AP-1 activity was increased by arginine, and the increase of AP-1 activity was decreased by SP600125 in IEC-6 cells: a vs. b $P < 0.01$, b vs. c $P < 0.01$, b vs. d $P < 0.01$, and c vs. d $P < 0.05$. **E** The induction of c-Jun activity by arginine was reduced by SP600125 in IEC-6 cells: a vs. b $P < 0.05$, a vs. c $P < 0.05$, b vs. c $P < 0.01$, b vs. d $P < 0.05$, and c vs. d $P < 0.05$

Fig. 3.

SP600125 reduced the increased iNOS expression in both in vivo and in vitro experiments.

A Animal experiments were performed as described in legend of Fig. 1. iNOS expression levels in intestine were measured by Western blot. Densitometry of bands is expressed in arbitrary densitometric units (lower panel). Values are represented as means $\pm$ SEM. *a* vs. *e* $P < 0.01$, *c* vs. *d* $P < 0.05$, *c* vs. *e* $P < 0.05$, *e* vs. *f* $P < 0.01$ ($n = 6$ for each group). **B** IEC-6 cells were treated with 10 mM of arginine (Arg), 20 μ M of SP600125 (SP), or vehicle for 24 h, and then were treated with H₂O₂ (250 μ M) for another 6 h. iNOS expression levels in cells were measured by Western blot. Densitometry of bands is expressed in arbitrary densitometric units (lower panel). Values are represented as means $\pm$ SEM: *a* vs. *b* $P < 0.01$, *b* vs. *c* $P < 0.01$, *b* vs. *d* $P < 0.05$, *c* vs. *d* $P < 0.05$ ($n = 3$ for each group)

Fig. 4. Silencing c-Jun by siRNA resulted in the decrease of iNOS expression. IEC-6 cells were treated with c-Jun siRNA for 48 h. Nuclear and cytoplasmic fractions in IEC-6 cells were separated, and Western blot and electrophoretic mobility shift assay were then performed. **a** Western blot analysis for the expression of c-Jun and iNOS. **b** Electrophoretic mobility shift assay for AP-1 DNA-binding activity

Fig. 5. 1400W did not affect the activity of c-Jun/AP-1. IEC-6 cells were treated with 10 mM of arginine (Arg), 20 μ M of special iNOS inhibitor 1400W, or vehicle (Ctrl) for 24 h, and then were treated with H_2O_2 (250 μ M) for another 6 h ($n = 3$ for each group). Densitometry of bands is expressed in arbitrary densitometric units (lower panel). Values are represented as means $\pm$ SEM. **A** Cell lysates were measured for p-c-Jun and c-Jun by Western blot. *a* vs. *b* $P < 0.05$, *a* vs. *c* $P > 0.05$, *b* vs. *d* $P > 0.05$. **B** Nuclear fraction was isolated and AP-1 DNA-binding activity measured by electrophoretic mobility shift assay: *a* vs. *b* $P < 0.05$, *a* vs. *c* $P > 0.05$, *b* vs. *d* $P > 0.05$.