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Arginine decreases peroxisome proliferator-activated receptor- γ activity via c-Jun

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Abstract

We have previously shown in the post ischemic gut that enteral arginine enhanced injury and inflammation via c-Jun/ AP-1 and abrogated peroxisome proliferator-activated receptor (PPAR) γ activity. In the current study, we investigated the mechanism by which arginine inhibited PPAR γ in vitro in rat small bowel epithelial IEC-6 cells. Arginine repressed PPAR γ transcriptional activity in a time and dose dependent fashion. Furthermore, downregulation of PPAR γ by arginine involved phosphorylation of c-Jun that occurred prior to changes in PPAR γ transcriptional activity. Silencing of c-Jun increased PPAR γ beyond that of nonsilenced cells and was not mitigated by arginine. Using a series of blocking studies, we found no relationship between arginine and the ligand-dependent binding site of PPAR γ . In conclusion, arginine decreased PPAR γ transcriptional activity in small bowel intestinal epithelial cells. These changes are due, in part, to phosphorylation of c-Jun and may explain the deleterious effects of enteral arginine in the post ischemic gut.

Keywords

Arginine; c-Jun; peroxisome proliferator-activated receptor- γ

Introduction

L-arginine (arginine) is a conditionally essential amino acid but becomes essential in disease states [1–3]. It plays an important role in cell growth, proliferation, migration, and particularly immune function [4, 5]. In the intestine, arginine accelerates intestinal mucosal regeneration, enhances bacterial clearance, and reduces bowel necrosis [6]. A recent meta-analysis demonstrated a significant decrease in post operative infections in patients receiving supplemental arginine prior to major surgery [7]. Ochoa et al have demonstrated that myeloid suppressor cells secrete arginase, leading to an arginine deficient state, and subsequent repressed T cell function [8, 9]. Arginine supplementation restored T cell function and lessened infectious complications. However, other investigators have suggested that critically ill patients, particularly those with sepsis, may not benefit or may even be harmed from arginine supplementation. Systemic inflammation may be intensified by arginine via inducible nitric oxide synthase (iNOS) [5, 10, 11]. We have shown in a rodent model of gut ischemia/reperfusion that enteral arginine increases gut injury and inflammation and enhances iNOS production [12].

Peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear receptor superfamily. PPARs are ligand-dependent transcription factors that bind to specific

peroxisome proliferator response elements (PPRE) at the enhancer sites of regulated genes [13]. PPAR γ plays a critical role in the immune response through suppression of inflammatory cytokines and to direct the differentiation of immune cells towards anti-inflammatory phenotypes [14].

The transcriptional activity of PPAR γ is regulated through several distinct mechanisms, including ligand-dependent transactivation, ligand-independent repression and ligand-dependent transrepression [15]. PPAR γ is a heterodimer transcription factor composed of PPAR γ and retinoid X receptor (RXR). The heterodimer is associated with the nuclear receptor corepressor complex in the absence of PPAR γ ligand. Upon activation by a ligand, the corepressor complex is replaced by coactivators leading to transcriptional initiation of target genes. The coactivators of PPAR γ include p300/ CREB binding protein (CBP), p160, PPAR γ coactivator-1, thyroid hormone receptor-associated protein 220 or PPAR γ -binding protein, androgen receptor-associated protein and PPAR γ -interacting protein. The corepressor for PPAR γ is a protein complex containing histone deacetylase 3 and silencing mediator for retinoic and thyroid hormone receptors or nuclear corepressor, and possibly, receptor-interacting protein [16].

PPAR γ is also a phosphoprotein. Its transcriptional activity is affected by kinases, notably the mitogen-activated protein kinases (MAPK), including both extracellular signal-regulated protein kinase (ERK) and the c-Jun NH₂-terminal kinase (JNK), but also protein kinase A and C, AMP kinase and glycogen synthase kinase-3 [17, 18].

We have shown in a rodent model of gut ischemia/reperfusion that arginine enhanced activator protein 1 (AP-1) via c-Jun but inhibited PPAR γ DNA binding activity [19]. AP-1 is a stress-activated DNA-binding protein composed of subunits that belong to the c-Fos and c-Jun families whose activities are regulated by MAPK. JNK phosphorylates a number of transcription factors, including the c-Jun component of the AP-1 transcription factor complex. c-Jun is involved in the regulation of diverse biological processes, including proliferation, inflammation, differentiation, apoptosis and cell migration [20, 21]. JNK can phosphorylate c-Jun on Ser63, Ser73, Thr91, and Thr93 within its transcriptional activation domain [22]. We have also shown in vitro that inhibition of JNK repressed arginine-mediated inflammation as well as c-Jun phosphorylation [23]. In the present study, we hypothesized that phosphorylation of c-Jun by arginine would inhibit PPAR γ transcriptional activity in a ligand-independent fashion.

Materials and Methods

Chemicals and reagents

The specific PPAR γ agonist, rosiglitazone, and antagonist, GW9662, were obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against c-Jun, phospho-c-Jun (Ser63) II (p-c-Jun) and β -actin were from Cell Signaling (Danvers, MA, USA). Antibody against PPAR γ was the product of Santa Cruz (Santa Cruz, CA, USA). ECL anti-rabbit IgG, horseradish peroxidase linked whole antibody (donkey) and ECL plus Western blotting detection system were purchased from GE Healthcare Bio-sciences Corp (Piscataway, NJ, USA). Dual-luciferase reporter assay system was ordered from Promega Corporation (Madison, WI, USA). Lipofectamine 2000 and Dulbecco's modified Eagle's medium (DMEM) and DMEM without arginine were from Invitrogen (Carlsbad, CA, USA). Arginine and D-arginine were the products of Sigma (Milwaukee, WI, USA).

Cell line

The rat small intestinal cell line, IEC-6, was purchased from the American Type Culture Collection and cultured using Dulbecco's modified Eagle's medium (DMEM), supplemented

with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

Western blot

IEC-6 cells were lysed with radioimmunoprecipitation assay buffer containing protease inhibitors (Sigma, Milwaukee, WI, USA). Proteins in the cell lysates were separated by electrophoresis on sodium dodecyl sulfate -polyacrylamide gel and transferred onto a Hybond-P membrane (Amersham Biosciences). The membrane was blocked with 5% fat-free milk in tris buffered saline with 0.1% Tween 20 for 1 h at room temperature, and incubated overnight at 4°C with antibody against phospho-c-Jun (Ser63), c-Jun, PPAR γ or β -actin. The blot was 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 film. The immunoblot band was quantified by densitometry and normalized to β -actin. The relative expression value in the control group was designated as 1.

RNA silencing of c-Jun

Pre-designed rat c-Jun siRNA (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'-GGCACAGCUUAAACAGAAAtt-3'. Interference transfections were performed with the silencer siRNA Starter Kit according to the manufacture's instructions.

Isolation of total RNA and real-time PCR

Total RNA was extracted from cells by the TRIzol isolation method according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using an iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturers' instructions. Primers for rat β -actin (Rn00667869-m1) and PPAR γ (Rn00440945_m1) were obtained from Applied Biosystems (Austin, TX, USA). Primer for rat c-Jun was: 5'-CGGCCCCGAACTTCTG-3'(forward) and 5'-GTCGTTTCCATCTTTGCAGTCA-3' (reverse) [24]. Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems, Austin, TX, USA). Gene expression was normalized against that of β -actin. The value for control cells was set to 1 and the value for the various treatments was presented as a fraction of this number.

Luciferase reporter gene assay

The luciferase reporter construct pGAL3 Basic or PPRE X3-TK-Luc (Addgene, Cambridge, MA, USA) [25] was transiently co-transfected into c-Jun siRNA transfected or untransfected IEC-6 cells at approximately 95% confluency with the Renilla control vector using Lipofectamine 2000. The PPRE-X3-TK-Luc reporter vector contains three copies of the PPRE upstream of the thymidine kinase promoter-luciferase fusion gene. After 24 h of transfection, cells were cultured in DMEM without arginine and FBS overnight. Cells were then treated with arginine (0–20 mM based on previous studies with glutamine [26] and cultured for indicated time points. For PPAR γ agonist and antagonist treatment, 10 µM of GW9662 (maximum effect on inhibition PPAR γ in IEC-6 cells, data not shown) and rosiglitazone [27], or vehicle was added to cells 1 h prior to arginine treatment. Subsequently, total cell lysates were prepared using 1X passive lysis buffer. Luciferase and Renilla activities were determined using a dual luciferase assay system in a Turner Designs TD-20/20 luminometer (Turner Designs) according to manufacturer's instructions.

Background values from pGL3 basic transfected cells were subtracted from all luciferase and Renilla readings. The luciferase values were then normalized to Renilla values.

Data Analysis

Statistical analysis was performed 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. Data are expressed as mean $\pm$ SEM and all experiments were performed in triplicate.

Results

Arginine increased phospho-c-Jun in small bowel intestinal epithelial cells

To validate our in vivo finding that arginine increased intestinal c-Jun [19], we examined c-Jun and phospho-c-Jun expression after arginine administration in small bowel intestinal epithelial cells. A time and dose-dependent increase in phospho-c-Jun expression by arginine was demonstrated (Fig. 1A and B). Expression increased by 1 h, returned to baseline by 8 h, and was maximal after 10 mM of arginine. To confirm that the increase in phospho-c-Jun was specific, expression in response to D-arginine, an inactive form of arginine [28, 29], was also measured. As shown in Figure 1C, D-arginine did not increase phosphorylation of c-Jun (Fig. 1C).

PPAR γ transcriptional activity is decreased by arginine

To gain preliminary insight into the role of arginine as a potential PPAR γ antagonist, the expression of PPAR γ in response to arginine stimulation was analyzed. There was no change in PPAR γ mRNA over time by arginine confirmed by quantitative PCR (data not shown). Immunoblotting similarly revealed no change in protein expression (data not shown). However, time course and dose response experiments showed that PPAR γ transcriptional activity decreased at two hours, remained repressed through 8 h, and was maximal after 10 mM of arginine (Fig. 2A and 2B). Importantly, the decrease in PPAR γ transcriptional activity occurred at later timepoints (Fig. 2B) than the increase in phospho-c-Jun (Fig. 1B).

Repression of PPAR γ activity by arginine is not via the ligand-dependent binding site

To determine if arginine was regulating the transcriptional activity of PPAR γ via ligand-dependent transrepression [15], the PPAR γ binding site was either activated by ligand or blocked by antagonist prior to arginine administration. Rosiglitazone is a thiazolidinedione that is a high affinity synthetic ligand of PPAR γ , whereas GW9662 (2-chloro-5nitrobenzanilide) is an irreversible antagonist that covalently modifies a cysteine residue in the ligand binding domain of PPAR γ [30, 31, 32]. Western blot analysis demonstrated that phospho-c-Jun expression was not altered by either Rosiglitazone (10 μ M) or GW9662 (10 μ M) (data not shown), suggesting that c-Jun did not affect the ligand-dependent binding domain of PPAR γ in small bowel epithelial cells and PPAR γ is the downstream of c-Jun.

Cells were next treated with either arginine, GW9662, or both, and PPAR γ transcriptional activity measured. Activity was significantly decreased by either arginine or GW9662, but the combined treatment further significantly decreased activity below that of either alone (Fig. 3), consistent with a mechanism that does not involve arginine binding to the ligand dependent binding site.

Arginine has no effect on PPAR γ activity in c-Jun silenced cells

The amino terminal A/B domain of PPAR γ contains a ligand-independent activation function (AF-1) which is phosphorylated at a consensus MAPK site, including Jun NH₂-terminal kinase (JNK) [33]. JNK then phosphorylates a number of transcription factors, including the c-Jun component of the AP-1 transcription factor complex. Our current findings suggested arginine decreased PPAR γ activity by phosphorylation of c-jun. To confirm these findings, a small RNA interference approach was utilized. c-Jun expression was silenced by 81.5% at the mRNA level (Fig. 4A) and 82.4% at the protein level (Fig. 4B). PPAR γ transcriptional activity in c-Jun silenced cells was significantly enhanced (Fig. 4C), showing that PPAR γ is the target of c-Jun activation.

Importantly, PPAR γ transcriptional activity was not repressed by arginine in c-Jun silenced cells (Fig. 5). Additionally, inhibition of the ligand dependent binding site by GW9662 decreased transcriptional activity to that of nonsilenced cells and activity was not restored by arginine (Fig. 5). These studies suggested that inhibition of PPAR γ activity by arginine is at the ligand-independent activation site.

Discussion

In the current study, we demonstrated that arginine repressed PPAR γ transcriptional activity in small bowel epithelial cells in a time and dose dependent fashion. Furthermore, downregulation of PPAR γ by arginine involved phosphorylation of c-Jun that occurred prior to changes in PPAR γ transcriptional activity. Silencing of c-Jun increased PPAR γ activity beyond that of nonsilenced cells and was not mitigated by arginine. Using a series of blocking studies, we found no relationship between arginine and the ligand-dependent binding site of PPAR γ .

There has been little investigation into the regulation of PPAR γ by arginine. Liu et al reported that arginine supplementation in a sepsis model increased PPAR γ mRNA in the intestines of pigs [34]. The reason for the differences between Liu's findings and the current study are unclear, particularly when Zigarelli et al and others have demonstrated inhibition of PPAR γ in sepsis [35, 36]. We have also shown inhibition of intestinal PPAR γ by arginine in the post ischemic gut [12, 19].

Nuclear receptors in general, and PPAR γ in particular, can be activated by ligand-dependent and independent mechanisms. The synergistic repressive effect of GW9662 and arginine on PPAR γ activity suggested that arginine may regulate the activity of PPAR γ , at least in part, via a ligand-independent manner. Activation by ligand-independent mechanisms typically involves kinase-dependent processes. PPAR γ is a phosphoprotein and MAPKs, like JNK, decrease its transcriptional activity [33, 37]. The phosphorylation site on PPAR γ by JNK has been mapped to a site with both ligand-dependent and independent functions [33]. As suggested by Camp et al [38], phosphorylation of PPAR γ by the JNK pathway could reduce the effectiveness of activating ligands. We have shown that glutamine activates PPAR γ via endogenous ligands [39] but when administered with arginine to intestinal epithelial cells, the profound protective effects of glutamine were significantly abrogated (unpublished observations).

The mechanism by which c-Jun regulates the activity of PPAR γ is not completely understood. CBP and its homologue p300 are transcriptional co-activators of various sequence-specific transcription factors including PPAR γ . The two homologous cofactors p300 and CREB binding protein have been shown to co-activate the ligand-dependent and -independent transcriptional activities of PPAR γ [40] and can interact with c-Jun [41, 42]. Thus, it is possible that c-Jun may compete with p300 and CREB binding protein to

suppress the activity of PPAR γ . Another possibility is that c-Jun via AP-1 exerts its suppression of PPAR γ . Fu et al reported that AP-1 markedly inhibited PPAR γ promoter activity in transient-transfection studies in vascular smooth muscle cells [43].

In conclusion, arginine decreased PPAR γ transcriptional activity in small bowel intestinal epithelial cells. These changes are due, in part, to phosphorylation of c-Jun and may explain the deleterious effects of enteral arginine in the post ischemic gut [12, 19].

Acknowledgments

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Abbreviations

AP-1	Activator protein 1
CBP	CREB binding protein
DMEM	Dulbecco's modified Eagle's medium
ERK	Extracellular signal-regulated protein kinase
FBS	Fetal bovine serum
iNOS	Inducible nitric oxide synthase
JNK	c-Jun NH ₂ -terminal kinase
MAPK	Mitogen-activated protein kinases
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response elements
RXR	Retinoid X receptor

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Fig. 1A

Fig. 1B

Fig. 1C **Fig. 1.**

Arginine upregulated phospho-c-Jun (p-c-Jun) expression in dose-response and time-dependent fashions. A. Arginine upregulated p-c-Jun expression in a time-dependent fashion. IEC-6 cells were cultured overnight in serum and arginine free medium then treated with 10 mM arginine for the indicated timepoints and p-c-Jun expression measured by Western blot analysis. B. Arginine upregulated p-c-Jun expression in a dose-response fashion. IEC-6 cells were cultured overnight in serum and arginine free medium then treated with indicated concentrations of arginine for 2 h and p-c-Jun expression measured by Western blot. C. D-Arginine (D-Arg) did not affect p-c-Jun expression. Cells were treated with 10 mM arginine (Arg) or D-Arg for 2 h then p-c-Jun expression measured by Western blot. Values represent the mean $\pm$ SEM. of 3 independent experiments per group.

Fig. 2A

Fig. 2.

Arginine downregulated PPAR γ activity in dose-response and time-dependent fashions. A. Arginine downregulated PPAR γ activity in a time-dependent fashion. IEC-6 cells were co-transfected the luciferase reporter construct pGAL3 Basic or PPRE X3-TK-Luc with the Renilla control vector for 24 h. Cells were cultured overnight in serum and arginine free medium then treated with 10 mM of arginine for indicated time points. PPAR γ activity was then evaluated by luciferase reporter gene assay. B. Arginine downregulated PPAR γ activity in a dose-response fashion. IEC-6 cells were co-transfected the luciferase reporter construct pGAL3 Basic or PPRE X3-TK-Luc with the Renilla control vector for 24 h. Cells were cultured overnight in serum and arginine free medium then treated with indicated concentrations of arginine. After 8 h, PPAR γ activity was evaluated by luciferase reporter gene assay. Values represent the mean $\pm$ SEM of 3 independent experiments per group.

Fig. 3.

Arginine and GW9662 had synergistic effects on downregulation of PPAR γ activity. Cells were co-transfected the luciferase reporter construct pGAL3 Basic or PPRE X3-TK-Luc with the Renilla control vector for 24 h and then incubated without FBS and arginine overnight. Cells were pre-treated with 10 μ M GW9662 (GW) or vehicle for 1 h and then added 10 mM arginine in indicated wells for 8 h. PPAR γ activity was evaluated by luciferase reporter gene assay. Values represent the mean $\pm$ SEM of 3 independent experiments per group.

Fig. 4.

Silencing of c-Jun resulted in the increase of PPAR γ activity. A. c-Jun mRNA was silenced by 81.5%. After transfection with c-Jun siRNA for 24 h, quantitative real-time PCR was performed for c-Jun mRNA expression. B. c-Jun protein expression was silenced by 82.4%. After transfection with c-Jun siRNA for 24 h, Western blot analysis was performed for c-Jun and PPAR γ protein expression. C. Transfection of c-Jun siRNA increased PPAR γ transcriptional activity. After transfection with c-Jun siRNA for 24 h, PPAR γ activity was evaluated by luciferase reporter gene assay. Bars represent the mean $\pm$ SEM of 3 independent experiments per group.

Fig. 5.

PPAR γ transcriptional activity was not significantly repressed by arginine in c-jun silenced cells. siRNA transfected cells were co-transfected the luciferase reporter construct pGAL3 Basic or PPRE X3-TK-Luc with the Renilla control vector for 24 h and then incubated without FBS and arginine overnight. Cells were then treated with 10 mM arginine (Arg) and/or 10 μ M GW9662 (GW) for 8 h. PPAR γ activity was then evaluated by luciferase reporter gene assay. Values represent the mean $\pm$ SEM of 3 independent experiments per group.