Fatty Acid-Bearing Albumin Induces VCAM-1 Expression through c-Src Kinase-AP-1/NF- κ B Pathways: Effect of L-Carnitine

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Key Words

C-Src kinase • Fatty acid-bearing albumin • L-Carnitine • Tubular cells • Vascular cell adhesion molecule-1

Abstract

Background: Fatty acid-bearing albumin [FA(+) albumin] exerts more deleterious effects in tubular cells than albumin alone. We investigated the effect of FA(+) albumin on the vascular cell adhesion molecule-1 (VCAM-1) expression and elucidated the underlying signaling pathways. We further examined the effect of L-carnitine, since it was known to modulate intracellular fatty acid concentration. **Methods:** Activation of AP-1 and NF- κ B was assessed by electrophoretic mobility shift assay. Phosphorylation of protein kinase was examined by Western blot analysis. VCAM-1 mRNA and protein expression were measured by Northern blot analysis and cell ELISA. **Results:** FA(+) albumin induced VCAM-1 expression via activation of AP-1 and NF- κ B, which was mediated through activation of c-Src kinase, followed by MAP kinases (p38, ERK 1/2, JNK-1) and I κ B kinase and I κ B- α , respectively. Inhibitors of protein kinase C and tyrosine kinase, anti-oxidants and intracellular calcium chelator suppressed the FA(+) albumin-induced activation of c-Src kinase. L-Carni-

tine suppressed the FA(+) albumin-induced VCAM-1 expression via inhibition of c-Src kinase. **Conclusions:** VCAM-1 expression with activation of c-Src kinase-AP-1/NF κ B pathways might be one of the possible mechanisms that linked FA(+) albumin to tubulointerstitial injury. L-Carnitine might be beneficial in attenuating FA(+) albumin-induced tubular injury.

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Introduction

Tubulointerstitial injury has been recognized as a key process in the progression of renal disease. Accumulating evidence has shown that urinary protein plays an essential role in progression of tubulointerstitial injury [1]. Although there now seems little doubt about the role of urinary albumin on the tubulointerstitial injury, it has been proposed that the effect of urinary albumin is not directly due to the protein molecule itself, but due to the fatty acids bound to albumin [2–4].

These fatty acids are known to provoke such effects as altered cellular growth, disturbed metabolism, increased apoptosis and the release of lipid metabolites with immu-

nologic and biochemical properties that may be pathologically important [3]. However, information about the effect of fatty acid bearing albumin [FA(+) albumin] especially on the expression and/or activation of certain signaling molecules still remains limited.

Tubulointerstitial injury is advanced by interstitial inflammation, which is characterized by an interstitial infiltrate of macrophage [5]. Vascular cell adhesion molecule-1 (VCAM-1) plays an important role in mediating the inflammatory process by recruitment of mononuclear leukocytes including macrophages and T lymphocytes [6]. However, the effect of FA(+) albumin on the VCAM-1 expression has not been shown yet.

Transcription factor nuclear factor- κ B (NF- κ B) is an important regulator of various genes involved in immune and inflammatory responses and activator protein-1 (AP-1) is linked to inflammatory and growth responses. FA(+) albumin is known to induce tubular NF- κ B activation [7–9]. However, it is not clear yet whether FA(+) albumin induces AP-1 activation in proximal tubular cells. Furthermore, relationship between the activation of NF- κ B and that of AP-1 by FA(+) albumin has not been revealed yet. Src family protein kinase is known to participate in the relay of a variety of signals from the membrane to transducers of cell activation and gene expression selectively [10]. We postulated that c-Src kinase might contribute to the pathogenesis of FA(+) albumin-induced tubular injury. Therefore, we examined the effect of FA(+) albumin on the expression of VCAM-1 and some cell-signaling proteins such as c-Src kinase, MAP kinases (ERK 1/2, JNK-1, p38), I κ B kinase, I κ B- α , transcription factor AP-1 and NF- κ B.

In addition, we examined the role of protein kinase C, tyrosine kinase, oxidative stress and intracellular calcium in the FA(+) albumin-induced signaling pathway.

Fatty acids taken up by proximal tubular cells via albumin are released intracellularly during albumin degradation [11]. If intracellular fatty acid is elevated out of control, it may lead to harmful effects on tubular cell functions [12].

Carnitine plays an important role in lipid metabolism, acting as an obligatory cofactor for beta-oxidation of fatty acids by facilitating the transport of long-chain fatty acids across the mitochondrial membrane as acylcarnitine esters. Thus, carnitine may exert some effects via modulating intracellular fatty acid concentration [13]. Therefore, we went further to examine the effect of L-carnitine on the FA(+) albumin-induced VCAM-1 expression.

Methods

Fatty acid-bearing bovine serum albumin (fraction V, low endotoxin), staurosporine, calphostin, genistein, herbimycin A, N-acetylcysteine and tiron were obtained from Sigma Chemical Company (St. Louis, Mo., USA). Intracellular calcium chelator 1,2-bis-(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethylester (BAPTA-AM) was obtained from Molecular Probes (Eugene Oreg., USA). Double-stranded NF- κ B and AP-1 oligonucleotides were obtained from Promega (Madison, Wisc., USA).

Phosphospecific and total ERK 1/2, JNK-1, p38, c-Src, I κ B kinase antibodies and horseradish peroxidase-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, Mass., USA). Anti c-Jun, c-Fos, I κ B- α and actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). VCAM-1 monoclonal antibody was obtained from R&D Systems Inc. (Minneapolis, Minn., USA).

The pharmacologic inhibitors of p38 (SB203580), ERK 1/2 (PD 98059), JNK-1, and c-Src kinase (PP2) were obtained from Calbiochem (San Diego, Calif., USA).

Cell Culture and Conditioning

All experiments were performed using HK-2 cells, a proximal tubular cell line derived from a normal adult human kidney [14]. Cells were grown with keratinocyte serum-free medium supplemented with EGF (5 ng/ml) and bovine pituitary extract (40 μ g/ml). Concentration of fatty acid-bearing albumin [FA(+) albumin] used in our experiment (0–10 mg/ml) was based on the previous studies [15, 16].

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was performed by incubation of nuclear protein (7.5 μ g) with P^{32} -labeled AP-1 or NF- κ B oligonucleotides at room temperature for 20 min in binding buffer as previously described [17]. Double-stranded AP-1 or NF- κ B oligonucleotides was labeled with γ - P^{32} -ATP using T4 kinase. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gels in 0.25 $\times$ TBE buffer. The band intensities were quantified using a GS-710 densitometer and QuantityOne software (Bio-Rad, Hercules, Calif., USA).

Specificity of AP-1 and NF- κ B-binding activity was confirmed by competition assay.

Western Blot Assay

Cytosolic protein was prepared as previously described [18]. Equal amount of protein from whole cell lysates was separated by a 10% SDS-polyacrylamide gels, transferred to nylon membrane. Membranes were incubated for 2 h with primary antibody, followed by peroxidase-conjugated secondary antibody. Antibody-antigen complexes were detected with ECL plus chemiluminescence (Amersham Pharmacia Biotech, Arlington, Ill., USA).

Northern Blot Analysis

10 μ g of total RNA was electrophoresed through 1% agarose, 2.2 M formaldehyde denaturing gel with MOPS buffer, followed by capillary transfer to nylon membranes. The membranes were hybridized with 32 P-labeled cDNA probes at 65 °C overnight. cDNA probes specific for human VCAM-1 and GAPDH (glycer-

Fig. 1. Activation of NF- κ B and AP-1 by fatty acid-bearing albumin. **a** Proximal tubular cells were incubated with fatty acid-bearing albumin (5 mg/ml) for indicated times (0–4 h). Electrophoretic mobility shift assays was performed for NF- κ B and AP-1. Bar graph depicted densitometric quantification of 3 replicates. # $p < 0.05$ vs. control.

0.05 vs. control. **b** Proximal tubular cells were incubated for 30 min with various concentration of fatty acid-bearing albumin (0–10 mg/ml). Bar graph depicted densitometric quantification of 3 replicates. # $p < 0.05$ vs. control.

aldehyde-3-phosphate dehydrogenase) were labeled with ^{32}P using random primers (Megaprime™ DNA labeling system, Amersham International, England). cDNA probes specific for human VCAM-1 and GAPDH were made by reverse transcription-polymerase chain reaction (RT-PCR) method, as previously described [17].

Cellular ELISA

Total cellular expression of VCAM-1 was measured by enzyme-linked immunosorbent assay (ELISA) on fixed adherent cells as previously described [19]. In brief, 10^4 tubular cells were cultured on 96-well plates. After incubation with FA(+) albumin for 8 h, cells were incubated with VCAM-1 monoclonal antibody for 18 h at room temperature and washed three times. Peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Westgrove, Pa., USA) was applied and incubated at 37°C for 60 min. After washing, 100 μl of TMB/E substrate solution was added for color reaction. Optical density of each well was measured at 450 nm with an ELISA reader (Molecular Devices, Menlo Park, Calif., USA).

Statistical Analysis

Data were expressed as mean $\pm$ SE. Kruskal-Wallis test was used for comparison of more than two groups, followed by a Mann-Whitney U test for comparison using a microcomputer-assisted program with SPSS for Window 10.0 (SPSS Inc., Chicago, Ill., USA). $p < 0.05$ was considered significant.

Results

Fatty Acid-Bearing Albumin [FA(+) Albumin] Induced Activation of NF- κ B and AP-1

FA(+) albumin induced activation of NF- κ B and AP-1 maximally within 30 min and then activity was subsequently declined (fig. 1a). NF- κ B was activated maximally at the dose of 5 mg/ml FA(+) albumin (fig. 1b).

Fatty Acid-Free Albumin Had No Significant Effect on the NF- κ B and AP-1 Activation

To compare the effects of albumin with and without fatty acid on the NF- κ B and AP-1 activation, EMSA was performed. EMSA revealed that 30 min incubation with fatty acid-free albumin had no significant effect on the NF- κ B and AP-1 activation (fig. 2).

FA(+) Albumin-Induced AP-1 Activation Was Mediated through Activation of c-Src Kinase, Followed by Activation of Mitogen-Activated Protein Kinases

FA(+) albumin (5 mg/ml) was found to induce phosphorylation of c-Src kinase and mitogen-activated pro-

Fig. 2. Effect of fatty acid-free albumin on the NF- κ B and AP-1 activation. Proximal tubular cells were incubated with fatty acid-free albumin [FA(-) albumin, 5 mg/ml] and fatty acid-bearing albumin [FA(+) albumin, 5 mg/ml] for 30 min. Electrophoretic mobility shift assays was performed for NF- κ B and AP-1. Bar graph depicted densitometric quantification of 3 replicates. # $p < 0.05$ vs. control.

Fig. 3. Induction of c-Src kinase, MAP kinase (ERK 1/2, JNK-1, p38), c-Jun and c-Fos by fatty acid-bearing albumin. Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+) albumin, 5 mg/ml] for indicated times (0–30 min). Western blotting was performed. The bands for phospho-c-Src kinase, phospho-ERK 1/2, phospho-JNK-1, phospho-p38 kinase, c-Jun and c-Fos were normalized to those for c-Src kinase, ERK 1/2, JNK-1, p38 kinase, actin, respectively, for standardization. Bar graph depicted densitometric quantification of 3 replicates at 10 min. # $p < 0.05$ vs. control.

Fig. 4. Suppression of fatty acid-bearing albumin-induced AP-1 and NF-κB activation by c-Src inhibitor and MAP kinase (p38, ERK 1/2, JNK-1) inhibitors. Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+)] albumin for 30 min with or without pretreatment with c-Src kinase inhibitor (PP2, 25 μM), p38 inhibitor (SB203580, 25 μM), ERK 1/2 inhibitor (PD98050, 40 μM) and JNK-1 inhibitor (20 μM), respectively, for 30 min. Electrophoretic mobility shift assays was performed for AP-1 (**a**) and NF-κB (**b**). Bar graph depicted densitometric quantification of 3 replicates. # $p < 0.05$ vs. control, ## $p < 0.05$ vs. FA(+) albumin.

Fig. 5. Effects of c-Src kinase inhibitor and L-carnitine on the c-Src kinase, ERK 1/2 and c-Jun. Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+)] albumin for 10 min with or without pretreatment with c-Src kinase inhibitor (PP2, 25 μM), L-carnitine (5 mM), respectively, for 30 min. Western blotting was performed. The bands for phospho-c-Src kinase, phospho-ERK 1/2, c-Jun were normalized to those for c-Src kinase, ERK 1/2, actin, respectively, for standardization. Bar graph depicted densitometric quantification of 3 replicates. # $p < 0.05$ vs. control, ## $p < 0.05$ vs. FA(+) albumin.

Fig. 6. Induction of IκB kinase and degradation of IκB-α by fatty acid-bearing albumin and effect of c-Src kinase inhibitor and L-carnitine. **a** Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+) albumin, 5 mg/ml] for indicated times (0–30 min). Western blotting was performed. Western blot (**a**) was representative of two separate experiments. **b** Proximal tubular cells were incubated with FA(+) albumin for 10 min with or with-

out pretreatment with c-Src kinase inhibitor and L-carnitine. Western blotting was performed. The bands for phospho-IκB kinase (IKK) and IκB-α were normalized to those for IKK and actin. Bar graph depicted densitometric quantification of 3 replicates. Left and right scales showed the results of p-IKK and IκB-α, respectively. [#] $p < 0.05$ vs. control, ^{##} $p < 0.05$ vs. FA(+) albumin.

tein (MAP) kinases (ERK 1/2, JNK-1 and p38) within 10 min. c-Jun and c-Fos were also induced by FA(+) albumin (fig. 3).

To determine whether FA(+) albumin-induced AP-1 activation was mediated through activation of c-Src kinase and MAP kinases, we examined the effect of c-Src kinase inhibitor and MAP kinase inhibitors on the FA(+) albumin-induced AP-1 activation. c-Src kinase inhibitor (PP2, 25 μM), p38 inhibitor (SB203580, 25 μM), ERK 1/2 inhibitor (PD98059, 40 μM) and JNK-1 inhibitor (20 μM), were found to suppress the FA(+) albumin-induced AP-1 activation (fig. 4a).

To investigate whether the activation of c-Src kinase was located upstream of MAP kinase activation, we ex-

amined the effect of c-Src kinase inhibitor on the activation of ERK 1/2 and c-Jun. Western blot revealed that c-Src kinase inhibitor, PP2 suppressed the FA(+) albumin-induced activation of ERK 1/2 and c-Jun (fig. 5), suggesting that FA(+) albumin-induced AP-1 activation was mediated through activation of upstream c-Src kinase, followed by activation of downstream MAP kinases.

FA(+) Albumin-Induced NF-κB Activation Was Mediated through Activation of c-Src Kinase, Followed by Activation of IκB Kinase and Degradation of IκB-α

Phosphorylation of IκB kinase (IKK) and degradation of IκB-α are known to be involved in the process of NF-

Fig. 7. Suppression of fatty acid-bearing albumin-induced VCAM-1 mRNA, AP-1 and NF- κ B by tyrosine kinase inhibitors, protein kinase C inhibitors, intracellular calcium chelator and anti-oxidants. **a** Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+) albumin] for 4 h with or without pretreatment with curcumin (15 μ M), PDTC (25 μ M), tyrosine kinase inhibitor (genistein 50 μ M), protein kinase C inhibitor (staurosporine 300 nM), intracellular calcium chelator (BAPTA-AM 25 μ M) and anti-oxidants (tiron 10 mM, N-acetylcysteine 50 mM).

Northern blot was performed for VCAM-1. Bar graph depicted densitometric quantification of four replicates. # $p < 0.05$ vs. control, ## $p < 0.05$ vs. FA(+) albumin. **b, c** Proximal tubular cells were incubated with FA(+) albumin for 30 min with or without pretreatment with curcumin, PDTC, genistein, staurosporine, BAPTA-AM, tiron and N-acetylcysteine. Electrophoretic mobility shift assays were performed for AP-1 and NF- κ B. Bar graph depicted densitometric quantification of 3 replicates.

κ B activation [20]. We found that FA(+) albumin induced phosphorylation of IKK and degradation of I κ B- α within 10 min (fig. 6a).

To determine whether FA(+) albumin-induced NF- κ B activation was mediated through activation of c-Src kinase, we examined the effect of c-Src kinase inhibitor on the NF- κ B activation. c-Src kinase inhibitor (PP2) was found to inhibit the FA(+) albumin-induced NF- κ B activation (fig 4b).

To investigate whether the activation of c-Src kinase was located upstream of I κ B kinase activation, we examined the effect of c-Src kinase inhibitor on the phosphorylation of IKK and degradation of I κ B- α . Western blot revealed that c-Src kinase inhibitor, PP2 suppressed the FA(+) albumin-induced phosphorylation of IKK and degradation of I κ B- α (fig. 6b), suggesting the FA(+) albumin-induced NF- κ B activation was mediated through activation of upstream c-Src kinase, followed by activation of IKK and degradation of I κ B- α .

In addition, MAP kinase inhibitors suppressed the FA(+) albumin-induced NF- κ B activation as well (fig. 4b).

FA(+) Albumin-Induced VCAM-1 Expression via Activation of AP-1 and NF- κ B

FA(+) albumin increased the expression of both VCAM-1 mRNA and protein (fig. 7a; table 1). As expected, fatty acid-free albumin had no significant effect on the VCAM-1 protein (table 1).

To determine whether FA(+) albumin-induced VCAM-1 expression was mediated through activation of AP-1 and NF- κ B, we examined the effect of PDTC, a inhibitor of NF- κ B, and curcumin, a inhibitor of AP-1, on the FA(+) albumin-induced VCAM-1 expression. PDTC (25 μ M) and curcumin (15 μ M) inhibited the FA(+) albumin-induced VCAM-1 mRNA and protein expression (fig. 7a; table 1). As expected, PDTC and curcumin suppressed the FA(+) albumin-induced NF- κ B and AP-1, respectively (fig. 8a).

MAP kinase inhibitors also suppressed the FA(+) albumin-induced VCAM-1 protein expression (table 1). These data suggested that FA(+) albumin-induced VCAM-1 expression was mediated through AP-1 and NF- κ B.

c-Src kinase inhibitor, PP2 inhibited the FA(+) albumin-induced VCAM-1 mRNA and protein expression as well (fig 8b; table 1).

Oxidative Stress, Intracellular Calcium, Protein Kinase C and Tyrosine Kinase Were Involved in the FA(+) Albumin-Induced Activation of c-Src Kinase

Tyrosine kinase inhibitor (genistein 50 μ M, herbimycin A 1 μ M), protein kinase C inhibitor (calphostin 500 nM, staurosporine 300 nM), intracellular calcium chelator (BAPTA-AM, 25 μ M) and anti-oxidants (tiron 10 mM, N-acetylcysteine 50 mM), were found to suppress the FA(+) albumin-induced phosphorylation of c-Src kinase, and subsequently ERK 1/2 (fig. 9a).

As a consequence, these agents inhibited the FA(+) albumin-induced AP-1, NF- κ B (fig. 7b, c), VCAM-1 mRNA (fig. 7a) and protein expression (table 1). These agents suppressed the FA(+) albumin-induced degradation of I κ B- α as well (fig. 9b).

L-Carnitine Suppressed the FA(+) Albumin-Induced VCAM-1 Expression via Inhibition of c-Src Kinase

L-Carnitine (5 mM) inhibited the FA(+) albumin-induced activation of c-Src kinase and subsequently ERK 1/2 and c-Jun (fig. 5). Consequently, L-carnitine inhibited the FA(+) albumin-induced AP-1, NF- κ B (fig. 8a) and

Table 1. Effect of pharmacologic agents on the fatty acid-bearing albumin-induced VCAM-1 protein expression

	VCAM-1 (OD)
Control	0.150 $\pm$ 0.001
FA(-) albumin, 5 mg/ml	0.177 $\pm$ 0.008
FA(+) albumin, 5 mg/ml	0.308 $\pm$ 0.008 [#]
FA(+) albumin + PP2, 25 μ M	0.180 $\pm$ 0.012 ^{##}
FA(+) albumin + PDTC, 25 μ M	0.171 $\pm$ 0.007 ^{##}
FA(+) albumin + curcumin, 15 μ M	0.169 $\pm$ 0.011 ^{##}
FA(+) albumin + SB 20358, 25 μ M	0.175 $\pm$ 0.010 ^{##}
FA(+) albumin + PD 98059, 40 μ M	0.173 $\pm$ 0.011 ^{##}
FA(+) albumin + JNK-1 inhibitor, 20 μ M	0.174 $\pm$ 0.007 ^{##}
FA(+) albumin + genistein, 50 μ M	0.184 $\pm$ 0.018 ^{##}
FA(+) albumin + herbimycin A, 1 μ M	0.195 $\pm$ 0.017 ^{##}
FA(+) albumin + calphostin, 500 nM	0.174 $\pm$ 0.007 ^{##}
FA(+) albumin + staurosporine, 300 nM	0.159 $\pm$ 0.007 ^{##}
FA(+) albumin + tiron, 10 mM	0.181 $\pm$ 0.013 ^{##}
FA(+) albumin + NAC, 50 mM	0.188 $\pm$ 0.009 ^{##}
FA(+) albumin + BAPTA-AM, 25 μ M	0.185 $\pm$ 0.016 ^{##}
FA(+) albumin + L-carnitine, 0.1 mM	0.237 $\pm$ 0.014 ^{##}
FA(+) albumin + L-carnitine, 1 mM	0.187 $\pm$ 0.011 ^{##}
FA(+) albumin + L-carnitine, 5 mM	0.156 $\pm$ 0.010 ^{##}

Data represent the mean $\pm$ SEM of 3–4 experiments in triplicate. [#] p < 0.05 vs. control, ^{##} p < 0.05 vs. FA(+) albumin. VCAM-1 = Vascular cell adhesion molecule-1.

VCAM-1 mRNA (fig. 8b). L-Carnitine suppressed the expression of FA(+) albumin-induced VCAM-1 protein in a dose dependent manner (0.1–5 mM) (table 1).

Discussion

Fatty acids bound to albumin are being recognized as important mediators of renal tubulointerstitial injury. There is accumulating evidence that, fatty acids carried on albumin exert extra deleterious effects on proximal tubular cells on top of those of albumin alone. For example, fibronectin and chemoattractant production was induced by fatty acids carried on albumin but not by pure albumin [4, 21].

In keeping with this notion, we found that fatty acid-bearing albumin [FA(+) albumin] was a more potent inducer of NF- κ B and AP-1 than fatty acid-free albumin.

Our goal in this study was to investigate the effect of FA(+) albumin on the VCAM-1 expression and to elucidate the underlying signaling pathways including c-Src

Fig. 8. Effects of L-carnitine on the fatty acid-bearing albumin-induced AP-1, NF- κ B and VCAM-1 mRNA. **a** Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+) albumin] for 30 min with or without pretreatment with curcumin (15 μ M), PDTC (25 μ M), L-carnitine (5 mM), respectively, for 30 min. Electrophoretic mobility shift assays were performed for AP-1 and NF- κ B. Bar graph depicted densitometric quantification of 3 replicates. # $p < 0.05$ vs. control, ## $p < 0.05$ vs. FA(+) albumin. **b** Proximal tubular cells were incubated with FA(+) albumin for 4 h with or without pretreatment with c-Src kinase inhibitor (PP2, 25 μ M) and L-carnitine (5 mM), respectively, for 30 min. Northern blot was performed for VCAM-1. Bar graph depicted densitometric quantification of 3 replicates.

kinase, MAP kinases (ERK 1/2, JNK-1, p38), I κ B kinase, I κ B- α and activation of AP-1 and NF- κ B (fig 10).

In addition, we examined the role of protein kinase C, tyrosine kinase, oxidative stress and intracellular calcium in the FA(+) albumin-induced signaling pathway. We went further to examine the effect of L-carnitine on the FA(+) albumin-induced signaling pathway since L-carnitine was known to modulate intracellular fatty acid concentration.

FA(+) albumin is known to induce tubular NF- κ B activation [7–9]. Protein overload is reported to induce AP-1 activation in renal podocytes [22]. However, it is not clear yet whether FA(+) albumin induces AP-1 activation in proximal tubular cells as well. We found that FA(+) albumin induced activation of AP-1 as well as NF- κ B maximally within 30 min. Therefore, activation of tubular NF- κ B and AP-1 by FA(+) albumin might

be one of the major mechanisms underlying FA(+) albumin-induced tubulointerstitial injury. It is well known that activation of tubular AP-1 and NF- κ B plays an important role in the pathogenesis of tubular inflammation [23, 24].

Macrophage infiltration is seen at the onset of experimental animal models of proteinuria. It has been reported that fatty acid-carrying albumin induces the production of a lipid chemoattractant, resulting in macrophage infiltration. Furthermore, fatty acid deficient diets abolish the macrophage infiltration in experimental animal models of proteinuria [2–4].

Among various genes dependent on the activation of AP-1 and NF- κ B, we examined the expression of VCAM-1 gene because VCAM-1 was known to play an important role in mediating the inflammatory process by recruitment of mononuclear leukocytes including macrophages

Fig. 9. Suppression of fatty acid-bearing albumin-induced c-Src, ERK 1/2 and IκB-α by tyrosine kinase inhibitors, protein kinase C inhibitors, intracellular calcium chelator and anti-oxidants. **a** Proximal tubular cells were incubated with fatty acid-bearing albumin [FA(+) albumin] for 10 min with or without pretreatment with tyrosine kinase inhibitors (genistein 50 μM, herbimycin A 1 μM), protein kinase C inhibitors (staurosporine 300 nM, cal-

phostin 500 nM), intracellular calcium chelator (BAPTA-AM 25 μM) and anti-oxidants (tiron 10 mM, N-acetylcysteine 50 mM), respectively, for 30 min. Western blotting was performed. The bands for phospho-c-Src kinase, phospho-ERK 1/2, IκB-α were normalized to those for c-Src kinase, ERK 1/2, actin, respectively for standardization. Bar graph depicted densitometric quantification of 3 replicates. [#] p < 0.05 vs. FA(+) albumin.

and T lymphocytes [10]. It is well known that tubular VCAM-1 expression is increased in a variety of inflammatory renal diseases [25].

We found that FA(+) albumin increased the expression of VCAM-1 mRNA and protein via activation of AP-1 and NF-κB. These data implicated that VCAM-1 expression by FA(+) albumin might promote the recruitment of mononuclear cells into interstitium, favoring the tubulointerstitial inflammation and disease progression. In support for this notion, interstitial macrophage infiltration was found with increased VCAM-1 mRNA expression in protein-overload proteinuria rat model [5].

Src family protein kinase is known to play a crucial role in the chemokine gene expression by soluble fibronectin, chemical anoxia [26, 27] and especially oxidants injury in tubular epithelial cells [28]. Furthermore, Src family kinases are able to promote the activation of MAP kinase [18], which is known to be one of the important upstream pathway of AP-1 activation. Therefore, we postulated that Src family kinases might act as upstream signaling molecules mediating the FA(+) albumin-induced AP-1 activation.

We found that FA(+) albumin-induced AP-1 activation was mediated through activation of c-Src kinase, followed by activation of MAP kinases (ERK 1/2, JNK-1, p38). We also found that FA(+) albumin-induced NF-κB

Fig. 10. Proposed signaling pathways involved in fatty acid-bearing albumin-induced VCAM-1 expression.

activation was mediated through activation of c-Src kinase, followed by activation of IκB kinase and degradation of IκB-α. These results suggested that c-Src kinase played a central role in the signaling pathway that linked FA(+) albumin to the activation of AP-1 and NF-κB. Therefore, c-Src kinase might provide a selective intervention site to protect the renal injury associated with FA(+) albumin.

In addition, we found that FA(+) albumin-induced NF-κB activation was also mediated at least in part, through the activation of the MAP kinase as well, suggesting that there was a crosstalk between MAP kinase and NF-κB activation. These findings were consistent with those of previous reports that MAP kinase was closely related with NF-κB activation [29, 30].

It had been reported that protein overload was associated with oxidative stress [31, 32], intracellular calcium

[33, 34], protein kinase C [7–8, 33, 35] and tyrosine kinase [35, 36]. In our study, we demonstrated that they were involved in the activation of c-Src kinase by FA(+) albumin.

Our study is a short-term study which examines the acute effects of FA(+) albumin over hours. These acute effects may come into play in nephrotic syndrome, which is perhaps unique in its combination of raised fatty acid levels and low albumin levels, producing a high fatty acid load per albumin molecule. Further studies are needed to establish the in vivo counterparts of our findings.

Fatty acids taken up by proximal tubular cells via albumin are released intracellularly during albumin degradation [11]. Elevation of the intracellular fatty acid is reported to contribute to harmful effects in acute hypoxic proximal tubular injury [12].

L-Carnitine is a naturally occurring compound that facilitates the transport of fatty acids into mitochondria for beta-oxidation [13]. Thus, carnitine may exert some effects via modulating intracellular fatty acid concentration. L-Carnitine is known to attenuate free fatty acid-induced endothelial dysfunction, suppress oleic acid-induced membrane permeability transition of mitochondria, act as an anti-oxidant and prevents the accumulation of end-products of lipid peroxidation [37–39]. Furthermore, L-carnitine is reported to reduce myocardial injury after ischemia and reperfusion by counteracting the toxic effect of high levels of free fatty acids, which occurs in ischemia [40].

L-Carnitine is being used in a spectrum of diseases including angina, acute myocardial infarction, postmyocardial infarction, congestive heart failure, peripheral vascular disease, dyslipidemia, diabetes mellitus, chronic renal failure with hemodialysis [41].

Therefore, we examined the effect of L-carnitine. We found that L-carnitine inhibited the FA(+) albumin-induced activation of c-Src kinase and subsequently suppressed the AP-1, NF-κB and VCAM-1 expression, suggesting that L-carnitine might be beneficial in attenuating the FA(+) albumin-induced tubulointerstitial injury.

Beneficial effects of L-carnitine may be due to its anti-oxidant activity in addition to its metabolic effects, because our data suggest that oxidative stress is involved in the FA(+) albumin-induced c-Src activation. Additional study is needed to determine about how L-carnitine works in the inhibition of c-Src activated by FA(+) albumin.

In conclusion, our study could thus provide an insight into the intrinsic toxicity of the fatty acids bound to albumin and also provide further clues regarding therapeutic approaches to inhibition of the signal transduction pathways.

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