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Reno-protective Effects of Eicosapentaenoic Acid (EPA) Against PAN Induced Nephrosis in WKY Rats

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ABSTRACT

Eicosapentaenoic Acid (EPA) is an omega-3 fatty acid (polyunsaturated fatty acid) that has pleiotropic effects as hypolipidemic and anti-inflammatory actions. Podocytes injury in the renal glomeruli has been proposed as the crucial mechanism in the development of focal segmental glomerulosclerosis or nephrosis. The effect of EPA on Puromycin aminonucleoside (PAN) induced nephrosis was tested. EPA was administered daily for 28 days at a dose of 1 g kg⁻¹ b.wt. then PAN was injected intravenously at a dose of 6 mg/100 g of body weight followed by EPA for 6 days. PAN nephrosis induced increase in proteinuria, lipid profiles, podocytes proteins expression and immunolocalization. EPA induced decrease in proteinuria and lipid profiles induced by PAN nephrosis. Also, EPA induced significant down-regulation in expression of connexin 43 and synaptopodin. Moreover, EPA induced 50% decrease in glomerular cell adhesion induced by PAN nephrosis. Immunofluorescence shows expression of desmin and connexin 43 in rat glomeruli that increased by PAN and decreased by EPA. These findings collectively showed that EPA has reno-protective effect during inflammation.

Key words: Connexin 43, eicosapentaenoic acid, expression, intermediate filament proteins, PAN nephrosis, renal injury

INTRODUCTION

Eicosapentaenoic Acid (EPA) is one of the n-3 poly unsaturated fatty acids (PUFA) which are contained in fish oil (Calder, 2003). EPA has many pleiotropic effects among which is its anti-thrombotic (Alshatwi and Alrefai, 2007), hypolipidemic, anti-inflammatory and anti-mitogenic actions (Hosseini *et al.*, 2009; Rahbar *et al.*, 2007). Like nigella (Soliman *et al.*, 2009) and propolis (El-Kott and Owayss, 2008), EPA ameliorates immune response and has hepato-protective functions and improve insulin sensitivity (Alsaif, 2004). Recently, EPA has been shown to acts as an antioxidant (Kojda and Harrison, 1999) parallel with those reported for *Nigella sativa*, a wildly

used medicinal plant (Abdelmeguid *et al.*, 2011). That peliotropic function of EPA is reported in humans, animals and birds (Fatahnia *et al.*, 2007; Al-Daraji *et al.*, 2010).

Feeding of fish oil reduces *in vivo* production of interleukin-1 (IL-1), IL-6, Tumour Necrosis Factor (TNF) and IL-2 by peripheral blood mononuclear cells and reduces the response to endotoxin and to pro-inflammatory cytokines, resulting in increased survival rate (Endres *et al.*, 1989; Hagiwara *et al.*, 2005). It has been shown that dietary supplementation with EPA retards the disease progression in human and experimental renal diseases and retards renal injury progression in patients with IgA nephropathy (Donadio *et al.*, 1994). Fish oil containing EPA inhibited mesangial cell activation and proliferation, reduced proteinuria and decreased histological glomerular injuries (Grande *et al.*, 2000) and improved albuminuria in type 2 diabetic patients (Shimizu *et al.*, 1995).

As known the renal glomeruli contain visceral epithelial cells that known as podocytes which are the most essential part to maintain stability of glomerular structure in kidney (Ichimura *et al.*, 2003; Omary *et al.*, 2004; Yaoita *et al.*, 1995). Podocytes are highly specialized epithelium that controls the bulk flow of filtrate through the intracellular spaces. They are situated at the basement of glomeruli as the terminal element in ultrafiltration barrier (Fries *et al.*, 1989). Once podocytes are injured, they cannot be replaced by new ones and its injury is the starting of focal segmental glomerulo-sclerosis and final glomerular tuft destruction and then chronic renal diseases (Kriz *et al.*, 1998). Moreover, podocytes in renal glomerulus express unusual intermediate filament proteins (IFs) for visceral epithelial cells. IFs cytoskeleton is mainly composed of vimentin, nestin, desmin, synaptopodin and connexin 43. During infection or inflammation, tissues are injured and that is accompanied by changes in the expressions of intermediate filament proteins (DePianto and Coulombe, 2004). IFs proteins expression as desmin is increased during renal injury or nephrosis induced by Puromycin Aminonucleoside (PAN) and their expression is controlled by a fashion that is specific to each cell and stage of cell differentiation (Yaoita *et al.*, 2002; Zou *et al.*, 2006, 2007). Vimentin is found in mesenchyme, desmin in muscle, glial fibrillary, connexin 43 in glomeruli at the gap junction, nestin in neuroepithelial stem cells and synaptopodin in cytoskeleton. Synaptopodin is a protein that is essential for the integrity of podocytes cytoskeleton because synaptopodin-deficient mice showed impairment in recovery from LPS-induced renal nephritic syndrome (Yanagida-Asanuma *et al.*, 2007).

Focal segmental glomerulosclerosis is a model of nephritic syndrome that can be induced by PAN (Diamond and Karnovsky, 1986; Kihara *et al.*, 1995). Oxygen radicals can be produced during PAN nephrosis due to podocytes injury and is followed by proteinuria that occurred without clear explanation (Nosaka *et al.*, 1997). In recent immuno-fluorescence staining studies, a striking change of a gap junctional protein, connexin 43 in podocytes in PAN nephrosis was seen. Gap junctions mediate cell-to-cell communication in various tissues (Goodenough *et al.*, 1996; White *et al.*, 1995). They contain channels that connect neighboring cells, allowing the movement of molecules smaller than 1000 d such as ions, nutrients, metabolite and second messengers and maintain cell stability and ultra-filtration barriers. Up till now, little data are available about the correlation between EPA and PAN nephrosis. The aim of this study was to test the effects of EPA during PAN nephrosis on urinary protein levels, lipid profiles and expression of renal intermediate filaments proteins expressed in podocytes.

MATERIALS AND METHODS

Materials: Male WKY rats 7 weeks age were purchased from Charles River Japan (Atsugi, Japan) and were used for these experiments. The following murine monoclonal antibodies were used: anti

Connexin 43 ((Sigma), anti-vimentin antibody (clone V9, Sigma, Saint Louis, MO, USA), anti-desmin antibody (clone D-33, Dako, Cytomation, Glostrup, Denmark). Anti-synaptopodin (Progen, Heidelberg, Germany). Eicosapentaenoic acid (EPA ethyl ester) was provided kindly by Mochida Pharmaceutical Co. Ltd Tokyo, Japan. Puromycin aminonucleoside (PAN) and Arabic gum was from Sigma Chemical Co., St. Louis, MO, USA.

Experimental design and PAN nephrosis induction: A total of 12 rats were used in this experiment, the experimental design was conducted from January, 2010 to November, 2010. Rats were divided into 2 groups, first group received EPADEL (EPA ethyl ester dissolved in Arabic gum) in a dose of 1 g kg⁻¹ body weight for 28 days by gavages. Second group was received Arabic gum 5% as control by gavages. At day 28, PAN nephrosis was induced in all groups by single intravenous injection of PAN (Sigma, USA) at a dose of 6 mg/100 g of body weight in phosphate-buffered saline. Following PAN injection, rats received EPA as treated group or Arabic gum as a control. Rats were housed in individual metabolic cages and their 24th urine specimens were collected at day 0, 2, 4 and 6 days after PAN injection. Rats were sacrificed under ether anesthesia. Blood samples were collected to extract serum and to measure changes in lipid profiles. Kidneys were removed and processed for western blotting, Immuno-fluorescence and histopathological examinations. Glomeruli were isolated from six rat kidneys and pooled and used as one sample of glomerular protein. The procedures for the present study were approved by the Animal Committee at Niigata, Zagazig and Benha Universities.

Proteinuria and lipid profiles measurements: Urinary protein excretion was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) according to manufacture's instructions. Blood samples were collected to extract serum and to measure lipid profiles using commercially available kits for total cholesterol, Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL).

Western blotting: Glomeruli were homogenized in lysis buffer (8 mol L⁻¹ urea, 1 mmol L⁻¹ dithiothreitol, 1 mmol L⁻¹ EDTA, 50 mmol L⁻¹ Tris-HCl, pH 8.0) with a sonicator on ice. Protein in sample was quantified by Lowry's method after precipitation by trichloroacetate with sodium deoxycholate as described previously (Zou *et al.*, 2006) with little modifications. Aliquot of 20 µg protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) under reducing conditions and proteins were electroblotted onto PVDF membrane (ImmobilonTM, Millipore, Bedford, MA, USA). The membrane was blocked for 2 h at room temperature in 5%(w/v) skimmed milk in 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01% Tween 20, followed by incubation with primary antibody (1:1000 dilution) of connexin 43, desmin, vimentin and synaptopodin overnight at 4°C. The membrane was washed 3 times with 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01% Tween 20 and incubated with 1:2000 diluted horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (Zymed laboratories, Inc. South San Francisco, CA, USA) for 1 h at room temperature. Visualization was performed using enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Renal histopathology: Small pieces of the kidney were collected from the WKY rats, kept overnight in methyl carnoa solution. The samples were cleared in ascending grades of alcohols, cleared in xylene and embedded in soft paraffin. Samples were cut at 5 µm thickness and then stained with PAS (Periodic Acid Schief), according to Bancroft *et al.* (1994).

Immuno-fluorescence microscopy: The indirect immuno-fluorescence technique was applied to frozen kidney sections and outgrowths from glomeruli as described previously by Yaoita *et al.* (2002) with little modification. In short, the rat kidneys were snap-frozen at -70°C , sectioned at a thickness of $3\ \mu\text{m}$ in a cryostat, fixed in 2% paraformaldehyde in PBS for 5 min and processed for double-label immunostaining. Outgrowths from explants cultured on eight-well glass chamber slides were fixed in methanol for 5 min, or fixed in 2% paraformaldehyde in PBS for 5 min permeabilized with 0.3% Triton \times -100 in PBS for 3 min and stained with antibodies specific for connexin 43 and desmin. For double-label immuno-fluorescence microscopy, rabbit anti-connexin 43 and anti-desmin antibodies (Sigma) and murine monoclonal antibody against ZO-1 (Zymed Laboratories, South San Francisco, CA) were mixed and applied as primary antibodies simultaneously. After washing with PBS, the sections were stained with fluorescein isothiocyanate-conjugated anti-rabbit IgG, rewashed with PBS and subsequently reacted with tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG. PBS, normal rabbit serum, or murine IgG1 monoclonal antibody (against rotavirus), shown not to react with rat glomeruli, were used as negative controls for the primary antibodies. Immunofluorescence of the sections and cultured cells were observed with an Olympus microscope (BX50) equipped with epi-illumination optics and appropriate filters, or with a laser scanning confocal microscope (MRC-1024; Bio-Rad Laboratories, Hercules, CA).

Statistical analysis: Results are expressed as Means \pm SE of 6 different rats. Statistical analysis was done using ANOVA and Fischer's post-hoc test using specific program (StatView Version-5; SAS Institute, Japan) for Macintosh computer. Significance was reported as $p < 0.05$.

RESULTS

Effect of EPA on urinary protein induced by PAN nephrosis: Measuring 24 h urinary protein secretion at day 0, 2, 4 and 6 revealed that PAN induced increase in urinary protein levels at day 4 and 6. Pretreatment of EPA to PAN injected rats significantly ($p < 0.05$) decreased the increase in urinary protein levels compared to PAN injected rats (control) at day 4 and 6 (Fig 1a).

Effect of EPA on PAN induced changes in lipid profiles in WKY rats: To test the renoprotective effects of EPA on PAN induced alteration in serum levels of cholesterol, LDL and HDL. Single injection of PAN induced significant increase in serum levels of cholesterol and LDL while decreased HDL levels, pretreatment of rats by EPA decreased PAN induced those changes, moreover, it decreased cholesterol and LDL levels and increased the levels of HDL as shown in Fig. 1b, d. Those findings are of biomedical importance for the pathogenesis of nephrosis induced by PAN.

Effect of EPA on PAN induced intermediate filament proteins expression: Using western blot analysis, IFs proteins expression was tested. As seen in Fig. 2, PAN increased protein expression of connexin43, synaptopodin and vimentin. Administration of EPA to WKY rats decreased PAN induced connexin43 and synaptopodin expression but not vimentin. So, EPA induced protective effect on PAN up-regulated connexin 43 and synaptopodin expression to maintain stability of Gap junction and decrease the incidence of nephrosis.

Effect of EPA on histopathology of kidney in PAN nephrosis: PAN injection induced massive intensive positive PAS materials outside capillary lumen and increase in cell adhesion of glomerular

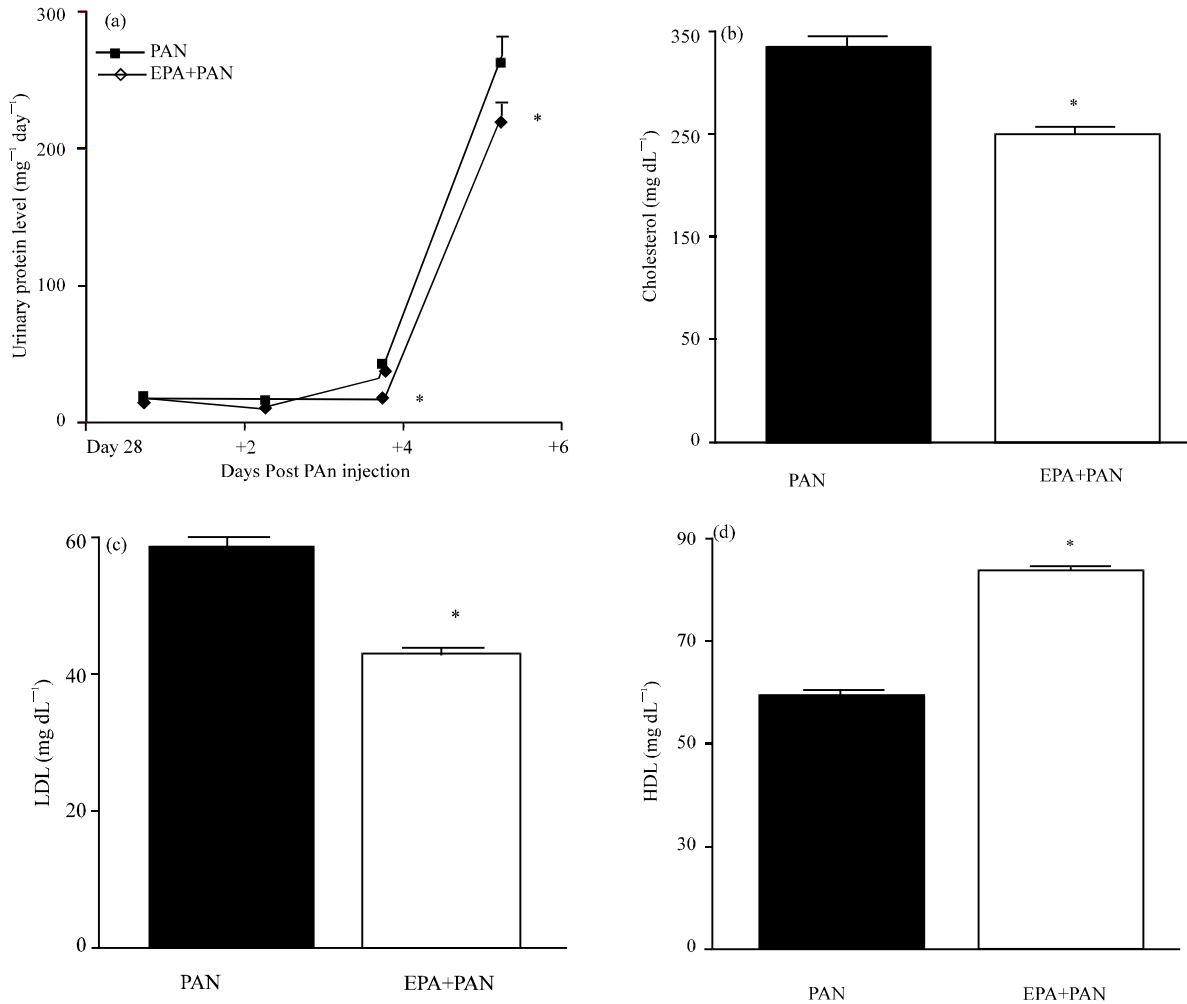


Fig. 1 (a-d): Effect of EPA on PAN induced changes in proteinuria and lipid profiles. EPA (mg kg⁻¹ bw⁻¹) or vehicle as control was administered to WKY rats for 28 day then PAN was injected intravenously (6 mg/100 g of body weight) and urine samples were collected at 0, 2, 4 and 6 days for changes in urinary proteinuria (a) WKY rats were killed at day 6 post PAN injection and the changes in cholesterol (b) LDL (c) and HDL (d) were tested Values are Means±SE of 5 different rats. *p<0.05 vs. PAN injected rats (control)

capillaries and Bowman capsules (Fig. 3A). EPA tended to decrease the changes in histopathology of kidney induced by PAN. It shows less adhesion in glomeruli and less intensive PAS materials outside capillary lumen (Fig. 3b). When the percentage of cell adhesion was counted, EPA significantly (p<0.05, 50%) decreases cell adhesion as seen Fig. 3c.

Effect of EPA on immunofluorescence expression of connexin43 and desmin in PAN nephrosis: Connexin 43 and desmin distribution and localization in the glomerulus were examined by double-label immunofluorescence microscopy using rabbit anti-Connexin 43 and anti-desmin antibodies (Fig. 4a, b) in combination with murine mono clonal anti-ZO-1 antibody (right pannel photo in (Fig. 4). Because the tight junction protein ZO-1 is concentrated in the intercellular

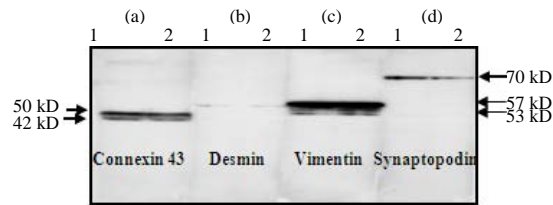


Fig. 2: Western blot analysis of intermediate filaments proteins expression separated from EPA (1) and PAN (2) treated WKY rats. Bands specific to each of the IF proteins are seen exclusively in renal glomeruli. Twenty milli grams protein was electrophoresed in 15% SDS-PAGE and electroblotted onto nitrocellulose membrane and detected by electro-detection after incubation with 1st and 2nd antibodies as written in materials. EPA induced inhibition in protein expression of connexin43 and synaptopodin but not desmin. Lane 1 is PAN treated rats; lane 2 is EPA treated PAN rats

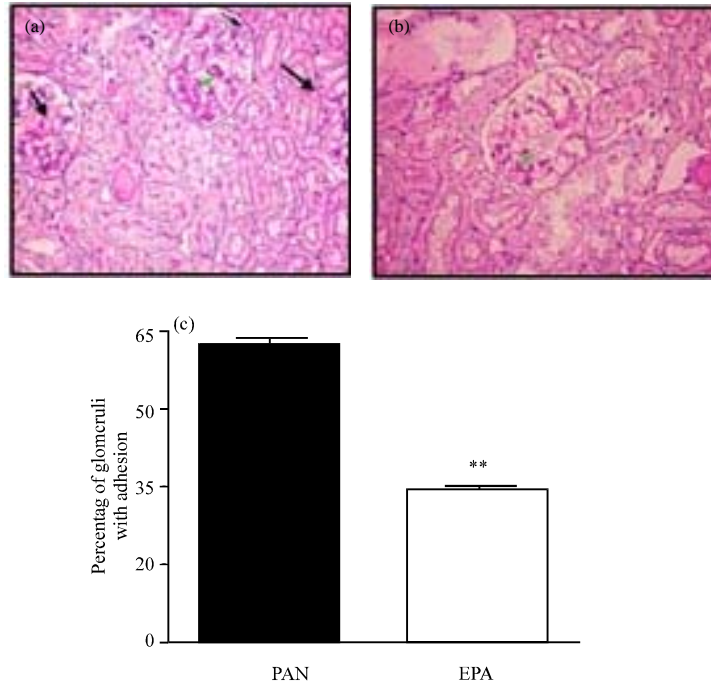


Fig. 3 (a-c): Effect of EPA on PAN induced changes in renal histology. (a) Photomicrograph of renal cortex from PAN treated rat groups showing intense positive PAS materials outside the capillary lumen (arrow head) and cellular adhesion between glomerular capillary pole and Bowman capsule (arrows) PAS (X40); (b) Photomicrograph of renal cortex from PAN pretreated with EPA groups showing normal glomeruli with less intense PAS positive materials outside the capillary lumen (arrow head) PAS (X40); (c) Percentage of cell adhesion from different 5 rat glomeruli, *p<0.05 vs. PAN injected rats (control)

junctions of podocytes under both normal and pathological conditions ZO-1 staining was used to locate the glomerular capillary wall. Normal kidney immunofluorescent dots for connexin 43 and desmin were observed mainly in the extra-glomerular mesangium and the neighboring intra-

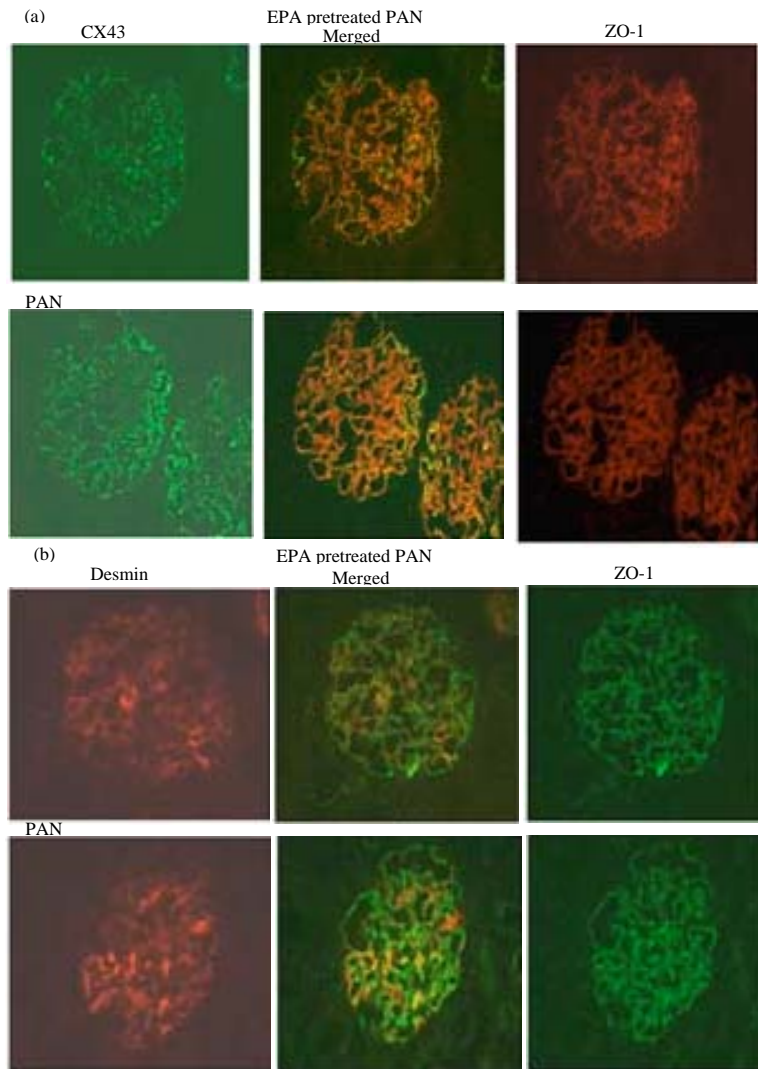


Fig. 4 (a-b): Double labeled immunofluorescence photomicrographs of frozen sections of rat kidneys incubated with antibodies against Connexin 43 (a) desmin (b) Co-localization of connexin43 and desmin and merged and ZO-1 (internal standard) in rat glomeruli. Magnifications is X100. Rabbit anti-Cx43, anti-desmin anti serum was detected with fluoresce in isothiocyanate-conjugated goat anti-rabbit IgG; mouse monoclonal anti-ZO-1 antibody was detected with tetramethylrhodam-ineisothiocyanate-conjugated goat anti-mouse IgG. EPA induced decrease in localization of connexin43 and desmin expression was seen

glomerular mesangium. Few but significant dots were also detected within glomeruli; most of them were located along the glomerular capillary wall. Administration of EPA to PAN injected rats induced significant decrease in connexin 43 and desmin expression in glomeruli as seen in down left of Fig. 4a, b. Those findings hypothesize a role for EPA during infection and inflammation and acts as anti-inflammatory agent against some destructive diseases as nephrosis.

DISCUSSION

In the present study, results showed the importance of EPA as protecting factors against inflammation in PAN nephrosis. Several relevant observations resulted from this analysis. First, EPA improved the renal effect of PAN on urinary protein secretion and in lipid profiles. Second, IFs proteins (Connexin 43, vimentin, synaptopodin) were expressed exclusively in the glomerulus, especially in the podocytes. Third, IF proteins were up-regulated in PAN nephrosis and inhibited by EPA administration. Recent studies have shown that dietary supplementation with n-3PUFA retards disease progression in non-diabetic renal diseases including IgA nephropathy (Donadio *et al.*, 1994) and EPA has a direct renal effect on PAN nephrosis. *in vitro* studies supported the assumption of direct renal effects of EPA (Hagiwara *et al.*, 2005). PAN nephrosis is widely used as a model of nephrotic syndrome progressing to focal segmental glomerulosclerosis (Doetsch *et al.*, 1999). A striking morphological feature in PAN nephrosis is the focal detachment of podocytes and that is coinciding with the onset of massive proteinuria (Ryan and Karnovsky 1975). Kim *et al.* (2001) reported that PAN injection caused a marked decrease in the podocyte number and an increase in the glomerular size. Because of the lack of cell proliferation, podocytes adapt to the decrease in cell number and glomerular growth by cell hypertrophy (Nagata and Kriz, 1992). As desmin staining in podocytes is either not detected *in vivo* under physiological conditions or is only weakly detected, the intense signals for desmin indicate the up-regulation of desmin in cultured podocytes (Yaoita *et al.*, 1995). These findings suggest the existence of an intimate relationship between IF proteins up-regulation and podocytes hypertrophy. Podocytes are generally attached to several capillaries by way of their foot and primary processes. Therefore, cell hypertrophy on enlarged glomeruli itself increases the mechanical stress to the entire cytoskeleton. The function of IFs is primarily to increase the mechanical resistance of cells (Omary *et al.*, 2004). It is therefore tempting to speculate that the up-regulation of IF proteins allow podocytes to progress to cell hypertrophy, which is suitable for glomerular growth but pretreatment with EPA inhibited that IFs expression induced by PAN to maintain renal barrier and normal renal function.

The timing of the up-regulation of IFs proteins in PAN nephrosis is differed among nestin, vimentin, and desmin (Yaoita *et al.*, 2002), although it is likely that the three IF proteins co-assemble together into mixed polymers. The transcriptional levels of connexin 43 and synaptopodin are already increased 6 days after PAN injection but the desmin transcripts was less detective and that is parallel with findings of Yaoita *et al.* (2002), who stated that desmin expression is 10 days after PAN nephrosis. Unlike, desmin is vimentin, its expression started at 3 days but in our study, its expression at 6 days and that the cause of less significance. The early morphological changes of podocytes in PAN nephrosis are mainly related with the foot processes, which are equipped with a complete microfilament-based contractile apparatus (Ichimura *et al.*, 2003). The loss or retraction of the foot process structure is noticeable by day 2 of PAN nephrosis (Ryan and Karnovsky, 1975) and that is clear in our findings as a decrease in cell adhesion was seen (Fig. 3). Moreover, Connexin 43 in glomeruli increased strikingly at both the protein and mRNA levels in PAN nephrosis, which is consistent with an important role for transcriptional regulation in the synthesis of Connexin 43 gap junctions.

Nephrotic Syndrome (NS) is characterized by proteinuria, oxidative stress and endogenous hyperlipidemia. Antioxidants and PUFA attenuate hypercholesterolemia related disturbances mainly because of their ability to reduce Reactive Oxygen Species (ROS) production and cholesterol, respectively (Kojda and Harrison, 1999). Moreover, omega-3 fatty acids from fish oil can reduce superoxide anion production by inflammatory cells. Free radical scavenging potential of both EPA

prevents ROS induced inflammation of hepatocytes in high cholesterol fed rats (Kumar *et al.*, 2006). The mechanism of EPA action on PAN nephrosis is unknown. It has been shown that synthesis of interleukin-1 and Tumour Necrosis Factor (TNF), both potent inflammatory factors, was suppressed by dietary supplementation with long-chain n-3 fatty acids (Endres *et al.*, 1989). Also, EPA suppressed the expression of TNF- α , an activator of NF- κ B, in human monocytes and prevented NF- κ B activation by preventing I κ B- α phosphorylation (Zhao *et al.*, 2004). This inhibitory effect of EPA on NF- κ B activation is thought to be mediated by the peroxisome proliferators activated receptors dependent pathway (Mishra *et al.*, 2004). As known, inflammation is the normal host response to infection or injury that mediates immune elimination of pathogens and tissue repair (Calder, 2003). The capacity of dietary n-3 polyunsaturated fatty acids (PUFAs) found in fish oil to suppress inflammation-associated processes has made them attractive candidates for both the prevention and amelioration of a variety of organ-specific and systemic diseases (Calder, 2008; Yaqoob, 2004).

CONCLUSION

EPA has reno-protective effects against PAN induced changes in urinary protein, lipid profiles, IF's proteins expression and suggest that other studies are needed to examine the mechanism by which EPA inhibited PAN nephrosis at the transcriptional levels.

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