ABSTRACT

Background: There is some evidence suggesting a close relationship between polyunsaturated fatty acids (PUFAs) and renal inflammation and fibrosis, which are crucial stages in chronic kidney disease.

Methods: To verify the role of PUFAs in renal fibrosis processes, we investigated the effects of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) on the gene expression of TGFβ, fibronectin (FN), connective tissue growth factor (CTGF) and type IV collagen (COLIV) in human mesangial cells, in the absence or presence of angiotensin II (AngII), using reverse transcriptase PCR.

Results: The addition of AA to mesangial cell cultures induced a significant up-regulation of TGFβ, FN, CTGF and COLIV expression, similar to that induced by AngII, while EPA and DHA had no stimulatory effects. The co-incubation of cells with AngII and AA potentiated AngII-induced gene expression; on the contrary, the co-exposure of cells to EPA or DHA suppressed the AngII- and AA-induced up-regulation of TGFβ, FN, CTGF and COLIV.

Conclusion: We conclude that the PUFAs have different effects, dependent on their chemical structure, on the AngII-TGFβ system, a major regulator of the renal fibrotic process. Our in vitro results may provide new therapeutic options toward interrupting the irreversible process of renal fibrosis and ameliorating chronic renal injury.

Key words: Angiotensin II, Arachidonic acid, Mesangial cells, n-3 PUFAs, Renal fibrosis, TGFβ

INTRODUCTION

A relationship between polyunsaturated fatty acids (PUFAs) and renal inflammation and fibrosis, crucial stages in chronic kidney disease, has been hypothesized (1). The beneficial effects of n-3 PUFAs observed in experimental and in human nephropathies could be due to their capacity to interfere with the synthesis of a variety of inflammatory factors and events, through mechanisms related both to the modulation of the balance of n-6-derived and n-3-derived eicosanoids and to the direct action on the cellular production of the major cytokine mediators of inflammation and on endothelium function (2-4). The mechanisms by which PUFAs can favorably interfere with some stages of renal fibrosis, characterized by mesangial cell activation and proliferation and extracellular matrix (ECM) production, include the activation of intracellular pathways leading to the regulation of the production of pro- and anti-fibrotic as well as pro- and anti-inflammatory factors (1).

In the present study we investigated the effects of different PUFAs on the gene expression of factors involved in renal fibrosis, such as TGFβ, fibronectin (FN), connective tissue growth factor (CTGF) and type IV collagen (COLIV) in cultured human mesangial cells (HMCs), in the absence or presence of the specific stimulator of mesangial cell activation, angiotensin II (AngII), which is recognized to play a key role in renal disease, because of its proinflammatory activity (5).
MATERIALS AND METHODS

Cell culture and treatment

Human mesangial cells (CC-2559) in primary culture were from BioWhittaker-Clonetics (Cambrex Proffarmaco, Milan, Italy). Cells were cultured in a humidified incubator at 37°C and 5% CO2, in RPMI 1640, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 ng/mL amphotericin-B, 2 mM L-glutamine, 16% heat-inactivated fetal calf serum (FCS; EuroClone, Celbio, Italy). When confluent, cells were washed twice with phosphate-buffered saline (Ca2+ and Mg2+ free), detached with a 0.025% trypsin-EDTA solution (Invitrogen, Milan, Italy) and expanded. Morphology and viability were evaluated by light microscopy using trypan blue exclusion. Cells were used at 80% confluence, passages 4-7. Twenty-four hours before treatment, FCS was reduced to 0.5%, and experiments were subsequently done in 0.5% FCS. The n-6 PUFA arachidonic acid (AA), and the n-3 PUFAs eicosapentaenoic acid (EPA; Cayman Chemical, Ann Arbor, MI, USA) and docosahexaenoic acid (DHA; Pronova BioPharma, Norway), were used at 50 µM for 1, 3, 6, 12, 24 and 48 hours. Experimental conditions were established by means of time- and dose-dependent (25, 50, 75 and 100 µM) experiments previously performed. Fatty acids were dissolved in ethanol, and control cells were treated with vehicle alone. At the end of the experiments, total RNA was extracted, and supernatants were collected. The experiments were carried out in the presence or absence of 10-7 M AngII (Sigma Aldrich, St. Louis, MO, USA), alone or in combination with fatty acids. None of the simultaneous treatments affected cell viability or morphology.

Gene expression

Gene expression of HMC-specific markers, regarding their secretion products (TGFβ, CTGF) and ECM components (FN, COLIV), was measured using the semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) approach, as previously described (6). At the end of the various treatments, total RNA was extracted using the OMNizol reagent. RNA quality was evaluated by Bioanalyzer 2100 (Agilent Technologies), and samples with RNA integrity number (RIN) >9 (7) were selected for use. Total RNA (1 µg) was used as template for cDNA synthesis (Applied BioSystems, Foster City, CA, USA). Each reverse transcription product was diluted 1:10 and stored at -20°C until used. Amplification was performed in a thermal cycler (MJ Research, Watertown, MA, USA) utilizing Jump-START Taq polymerase (Sigma Aldrich, St. Louis, MO, USA), specific primers for the target genes.

Fig. 1 - Effects of angiotensin II and fatty acids on TGFβ, FN, CTGF and COLIV gene expression in human mesangial cells (HMCs). TGFβ, FN, CTGF and COLIV gene expression, measured by RT-PCR, in primary HMCs treated with 10-7 M AngII or 50 µM fatty acids for 12 and 24 hours. The mRNA levels are reported as target gene to 18S ratio and normalized to control. Data represent the means ± SD of 3 experiments performed in duplicate. Statistical analysis was carried out using a between-within ANOVA design. *p≤0.05, #p≤0.005, §p≤0.0005, vs. control (basal condition).
and 18S rRNA as housekeeping gene. Amplification products were analyzed after polyacrylamide gel electrophoresis and silver staining. Quantification was performed by densitometric analysis (NIH Image Analyzer software).

**Protein expression**

Production of TGFβ was determined in the culture supernatants using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, with a limit of detection <7.0 pg/mL (R&D System, Abingdon, UK).

**Statistical analysis**

Data represent means ± SD of 3 different experiments performed in duplicate. Results are expressed as the ratio between the level of target gene expression following different treatments and the level of expression obtained in controls. Statistical analysis was carried out by ANOVA with a between-within design and by Student’s t-test, as specified.

**RESULTS**

Exposure of cultured HMCs to n-6 and n-3 PUFAs had specific, chemical structure–related effects on the gene expression of TGFβ, FN, CTGF and COLIV and on TGFβ protein secretion. Differences in the behavior of PUFAs in modulating the expression of the profibrotic factors were observed in mesangial cells both in basal conditions and after stimulation with AngII.

RT-PCR analysis demonstrated that after 12 and 24 hours of cell culture incubation, AA induced a significant up-regulation of TGFβ, FN, CTGF and COLIV gene expression, comparable to that reached upon stimulation with AngII. In contrast, under the same experimental conditions, EPA and DHA had no stimulatory effect (Fig. 1). Moreover, compared with those in the EPA-treated cell group, the levels of secreted TGFβ were significantly increased in cells incubated with AngII or AA after 24 and 48 hours (Fig. 2). In addition, when the cells were incubated simultaneously with AngII and 1 of the 3 PUFAs, AA caused a significant increase in the AngII-induced up-regulation of all 4 genes, while incubation with EPA and DHA resulted in a significant inhibitory effect (Fig. 3). The same suppressive action was demonstrated by EPA and DHA in experiments of coexposure with AA (Fig. 4).

**DISCUSSION**

The present investigation is the first to report that PUFAs determine specific, chemical structure–related modulation of AngII-induced inflammatory effects in cultured mesangial cells. In fact, the n-6 PUFA AA was as effective as AngII in up-regulating TGFβ, FN, CTGF and COLIV, while the n-3 PUFAs EPA and DHA had no stimulatory effects. These data were supported by the finding that TGFβ protein production paralleled its gene expression, and it is plausible that the protein secretion related to the other genes behaved similarly, given that CTGF is one of the downstream effectors of TGFβ. Interestingly, n-3 PUFAs suppressed both AngII- and AA-induced up-regulation.

Suppressive effects of fish oil, known to be rich in n-3 PUFAs, on mesangial cell proliferation (8), on platelet-derived growth factor–induced DNA synthesis in rat mesangial cells (9) and on mitogen-induced endothelin-1 production in bovine mesangial cells have been reported by several authors (10).

Recent studies conducted in our laboratory on osteoblastic cells have demonstrated that PUFAs have different and direct effects, dependent on their chemical structure, on the gene expression of some cytokines such as IL-1α, IL-1β, IL-6, TNFα and macrophage colony-stimulating factor,
as well as of inducible nitric oxide synthase, and on the adhesion cellular process (6, 11, 12).

On the whole, the most important finding of the present study is that PUFAs are able to interfere with the AngII-TGFβ axis, which plays a critical role in regulating the renal fibrotic process. Undoubtedly, AngII is a key factor in the progression of glomerular sclerosis and chronic kidney diseases, not only because it participates in the regulation of intraglomerular hemodynamic changes, but also because it is related to mesangial cell proliferation and ECM formation (5, 13-15). Upon stimulation with AngII, HMCs have been shown to overexpress a number of genes, such as TGFβ and plasminogen-activator inhibitor type-1, and to produce excess ECM components as FN, the accumulation of which is a hallmark of progressive glomerular disease (16). Our observation of up-regulation of TGFβ, FN, CTGF and COLIV genes induced by AngII confirms these literature data.

Interestingly, AngII in renal cells stimulates the synthesis of TGFβ, the fibrogenic factor that plays a central role in the development of glomerulosclerosis either directly, by stimulating the synthesis of ECM components, FN and COLIV, and reducing collagenase production (15, 17), or indirectly through other profibrogenic factors such as CTGF, which is the main downstream effector of TGFβ and is considered a molecular marker of the fibrotic response (16, 18).

The interaction between AngII and TGFβ and the participation of both in regulating the fibrotic process is very intriguing. Despite enormous complexities, several important issues have recently come to light. AngII and other members of the renin-angiotensin-aldosterone system activate the TGFβ axis by both indirect and direct mechanisms, involving transcriptional and post-transcriptional events and kinase-dependent pathways (19-22). Our study identifies AA on the one side and EPA and DHA on the other side as physiological profibrotic and antifibrotic factors, respectively, since we showed a modulatory activity on the AngII-induced expression of genes involved in the progression of renal disease. At present, we cannot indicate whether such modulation occurs through a direct action of EPA and DHA on each one of the genes evaluated or indirectly through their interaction with the AngII-TGFβ axis. Moreover, the biochemical mechanism explaining the suppressive effects of n-3 PUFAs on the AngII- and AA-induced expression of TGFβ and FN and COLIV is also unclear. It is likely that EPA and DHA interfere with, and share the same signal transduction pathways mediating, AA- and AngII-induced TGFβ synthesis. Consistent with this hypothesis are the data coming from the literature and from our own researches. Several lines of evidence suggest that the metabolic cascade of n-6 PUFAs might be modulated by n-3 PUFAs,
which can influence desaturase activity and induce a decrease in AA synthesis, thus interfering with the phospholipase C cascade (23). Moreover, PUFA constituents of cell membranes are recognized as second messengers and can determine the activation of some protein kinases, such as PKC and JNK, ERK and tyrosine kinases, thus interfering with the cellular events responsible for the development of renal fibrosis (24-26).

Interestingly, Camandola et al demonstrated that AA but not EPA activated nuclear factor kB (NF-kB) (27), an intracellular signaling molecule known to play a crucial role in AngII-induced inflammatory renal damage (28-30). Li and Zhuo recently reported that AngII-induced transcription of cytokines, chemokines and growth factors, targeting kidney injury, are primarily caused by angiotensin receptor (AT1)-activated NF-kB signaling (31). Further studies are needed to identify the mechanism responsible for the interaction between PUFAs and AngII-induced proinflammatory effects.

Whatever the biochemical mechanism underlying the effects of EPA and DHA on the expression of profibrotic factors in mesangial cells, a better understanding of how PUFAs modulate the renin-angiotensin-aldosterone system and TGFβ axis could lead to the development of innovative strategies to arrest or reverse fibrotic processes in kidney diseases.

Direct modulation of the TGFβ system is not as yet feasible in humans; angiotensin-converting enzyme inhibitors and AT1 blockers are currently the most effective drugs interfering with AngII-mediated TGFβ expression (32). The in vitro effects of EPA and DHA on the TGFβ axis provide new therapeutic implications and a possible clinical approach, utilizing natural substances such as fish oil to attenuate renal damage in kidney diseases. In addition, our results offer a rationale for the studies showing that dietary PUFA manipulation influences disease progression in human and experimental nephropathies (33).

Further in vivo and in vitro investigations are needed to make the intriguing possibility of interfering with AngII and TGFβ systems by means of natural and dietary components such as fish oil, more than a simple speculation.

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