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Unsaturated but not saturated fatty acids induce transcriptional regulation of CCL2 in pancreatic acini. A potential role in acute pancreatitis

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ABSTRACT

Fatty acids (FAs) are massively released from peripancreatic fat during acute pancreatitis (AP) and they were shown, as a whole, to induce inflammatory response in pancreatic acini. We investigated the mechanisms triggered by the major saturated FAs (SFAs) and unsaturated FAs (UFAs) in modulating the expression of chemokine (C–C motif) ligand 2 (CCL2) in acinar cells. Pancreatic acini of control rats were treated with palmitic acid (PA) or stearic acid (SA), as SFAs, or oleic acid (OA) or linoleic acid (LA), as UFAs. By using specific inhibitors, the involvement of MAPKs (JNK, ERK, p38), JAK, NF-κB and STAT3 pathways was assessed. The role of PPARγ pathway was studied by using 15-Deoxy-Δ(12,14)-prostaglandin J(2) (15d-PGJ2). CCL2 mRNA was analyzed by qRT-PCR. By western blot, phosphorylated forms of MAPKs and JAK as well as IκB-α were analyzed in cytoplasm and p65-NF-κB and phospho-STAT3 in nucleus. No effect was found in PA- or SA-treated acini. Conversely, in response to OA or LA, MAPKs and JAK acted as upstream signals, driving the CCL2 up-regulation transcriptionally mediated by the synergic action of NF-κB and STAT3. By blocking the activation of NF-κB and STAT3, 15d-PGJ2 totally inhibited the OA- and LA-induced CCL2 overexpression. We conclude that the most common UFAs, but not the SFAs, represented in peripancreatic fat and released during AP, are capable of up-regulating the acinar expression of CCL2, which depends on the activation of MAPK/JAK-mediated NF-κB and STAT3 pathways. By targeting both transcription factors, PPARγ agonists could be indicated as potential therapy in AP.

1. Introduction

Elevated concentration of free fatty acids (FAs) in plasma is a common feature in inflammatory diseases, including acute pancreatitis (AP) [1,2]. Regardless of AP etiology, digestive enzymes, such as lipases and phospholipase A2, are leaked out from the injured acinar cells into the pancreatic interstitium and blood stream [3]. As a result of the lipase action on peripancreatic adipose tissue, high amounts of FAs are generated, an event associated with multisystem organ failure during severe forms of AP [4,5] which is especially relevant in obesity. With regard to this, obesity is considered a risk factor that aggravates the progression of AP in humans [5–7] and animal models [8,9], although the underlying mechanisms are not fully understood.

Noxious effects have been attributed to FAs due to their cytotoxic action disrupting cell membranes [10], as well as to their role in modulating inflammation [11]. Currently, there is growing interest in investigating the involvement of these lipid derivatives in diverse inflammatory disorders; however, information about the cellular mechanisms associated with the FA response is incomplete. Contradictory effects have been reported for individual FAs on different cell types. Thus, pro-inflammatory [12] and no effects [13] have been found in response to saturated FAs (SFAs). Regarding unsaturated FAs (UFAs), anti-inflammatory [14], pro-inflammatory [13,15,16] and no effects [17] have also been reported. Controversial data may be explained because the response might be cell dependent and specific for each FA according to its chemical structure. So, it is important to assess the effects exerted by individual UFAs and SFAs on the same cell type as well as the underlying mechanisms to trigger the cell response.

The effects of different lipid components on pancreatic acini have not yet been thoroughly investigated despite the relevance they may have in the pathophysiology of AP. In a recent study [18], we reported that total lipids as well as the FA fraction obtained from necrotic adipose tissue of rats with severe AP up-regulated the expression of inflammatory mediators in pancreatic acini. Given that in peripancreatic fat necrosis of rats with AP, the most abundant SFAs proved to be palmitic acid (PA, C16:0) and stearic acid (SA, C18:0), and the major UFAs

Abbreviations: AP, acute pancreatitis; CCL2, chemokine (C–C motif) ligand 2; ERK, extracellular signal-regulated kinase; FA, fatty acid; JAK, Janus kinase; JNK, Jun NH2-terminal kinase; LA, linoleic acid; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-kappa B; OA, oleic acid; PA, palmitic acid; PPAR, peroxisome proliferator-activated receptor; SA, stearic acid; SFA, saturated fatty acid; STAT, signal transducer and activator of transcription; UFA, unsaturated fatty acid; 15d-PGJ2, 15-Deoxy-Δ(12,14)-prostaglandin J2

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were oleic acid (OA, C18:1) and linoleic acid (LA, C18:2) [18], the aim of the current study was to assess their individual role in triggering mechanisms that lead to the inflammatory response in acinar cells. As a representative inflammatory mediator with a key role in AP, we analyzed the expression of chemokine (C–C motif) ligand 2 (CCL2) in pancreatic acini exposed to the above-mentioned FAs and the underlying signaling pathways involved in the FA response. We show that the most abundant UFAs, but not the SFAs, in necrotic peripancreatic fat tissue of rats with AP activated molecular pathways which lead to CCL2 overexpression in acinar cells. The relevance of this study lies in furthering the understanding of the role of FAs in the pathophysiology of AP and in the approach of potential therapeutic strategies to the disease.

2. Materials and methods

2.1. Chemicals

Collagenase type XI, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), amino acids solution, bovine serum albumin (BSA), palmitic acid, stearic acid, oleic acid, linoleic acid, soybean trypsin inhibitor (STI), phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), digitonin, protease inhibitor cocktail, phosphatase inhibitor cocktail, Nonidet P-40, SP600125, AG-490 and 15-Deoxy-Δ(12,14)-prostaglandin J(2) (15d-PGJ2) were supplied by Sigma Chemical Co. (Madrid, Spain). SB202190 was from Calbiochem (Darmstadt, Germany). GDC-0994, SH-4-54 and JSH-23 were kindly donated by Selleckchem (Munich, Germany). Other standard analytical grade laboratory reagents were obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male Wistar rats (250–300 g body weight) were supplied by the experimental animal service of the University of Salamanca (Spain). They were housed individually in cages, maintained at 22 ± 1 °C using a 12 h light/dark cycle and fed with standard laboratory chow (Teklad-Harlan 2014, Mucedola, Milan, Italy) and tap water *ad libitum*. The animals were fasted overnight before the experiments but they were allowed free access to water. The study was performed in accordance with European Community guidelines (2010/63/EU) and approved by the Institutional Animal Care and Use Committee of the University of Salamanca (Spain).

2.3. Isolation and treatment of pancreatic acini

After 12 h fasting and under anesthesia with sodium pentobarbital (3 mg/100 g body weight, intraperitoneally), pancreas of control rats was removed in order to isolate pancreatic acini by digestion with collagenase as previously described [18]. Afterwards, pancreatic acini were resuspended in Na-HEPES solution (pH 7.4) enriched with 14 mM glucose and 1% essential aminoacid mixture and containing 1% (*w*/*v*) BSA, 0.01% (*w*/*v*) STI, and 0.5 mM $CaCl_2$ and incubated (5% CO_2, at 37 °C) for 1 h in the presence of the following FAs: PA (C16:0), SA (C18:0), OA (C18:1) and LA (C18:2) or the vehicle (EtOH) at concentrations ≤1% (controls). In some experiments, acini were pre-treated with SP600125 (50 μM), GDC-0994 (50 μM) or SB202190 (50 μM), as inhibitors of JNK, ERK and p38-MAPK, respectively; AG-490 (50 μM) as a JAK inhibitor; JSH-23 (30 μM), SH-4-54 (10 μM), as inhibitors of NF-κB and STAT3, respectively; or the vehicle dimethyl sulfoxide (DMSO) at concentrations ≤0.3%. In another set of experiments, the effect of PPARγ activation on the FA response was evaluated by incubating acinar cells with 15d-PGJ2 (10 μM), a specific PPARγ agonist, added 5 min after initiating the incubation in the absence or presence of each FA.

2.4. Lactate dehydrogenase (LDH) activity

LDH leakage was evaluated as a measurement of the cell damage. LDH activity was analyzed according to the method of Gutmann and Wahlefel [19]. Cellular LDH was measured after lysis of pancreatic acini with a 50 mM Tris–HCl buffer (pH 7.2) containing 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EDTA, 10 mM PMSF, 1 mM DTT and 150 μg/ml digitonin. The released LDH was measured in the incubation medium. Changes in absorbance due to β-nicotinamide dinucleotide$^+$ formation were recorded at 339 nm at 30 °C. We computed the total LDH and calculated the LDH leakage as percentage of the total measured inside and outside cells.

2.5. Western blot analysis of MAPKs, JAK, NF-κB and STAT3 activation

Cytoplasmic and nuclear extracts of pancreatic acini were obtained as previously reported [18]. Samples (30 μg) were individually separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Phospho-MAPKs (p-MAPKs), phospho-JNK (p-JAK) and IκB-α were analyzed in cytoplasm and p65 and phospho-STAT3 (p-STAT3) in nuclear extracts. Non-specific binding was blocked by incubating the blot in Tris-buffered saline (TBS) pH 7.6, containing 0.1% (*v*/*v*) Tween 20 and 5% (*w*/*v*) nonfat dry milk for 1 h. Afterwards, blots were incubated with the primary antibody against either each of the three p-MAPKs (p-JNK, p-ERK, p-p38), p-JAK, IκB-α, p65 or p-STAT3 (Cell Signaling Technology, Beverly, MA) at 1:1000 dilution in TBS buffer pH 7.6, containing 0.1% (*v*/*v*) Tween 20 and 5% (*w*/*v*) BSA overnight at 4 °C. α-Tubulin and lamin B1 (Cell Signaling Technology, Beverly, MA) were used as the load control of cytoplasmic and nuclear proteins, respectively. After washing with TBS containing 0.1% Tween 20, the blots were incubated for 1 h at room temperature with the respective horseradish peroxidase-conjugated secondary antibody at 1:2000 dilution in TBS buffer pH 7.6, containing 0.1% Tween 20 and 5% (*w*/*v*) nonfat dry milk and finally they were developed for visualization. The bands were detected with the Phototope-HRP Detection kit (Cell Signaling Technology, Beverly, MA). Image J 1.32 software from http://rsbweb.nih.gov/ij/download.html was used to quantify the intensity of the bands. Relative protein levels were calculated compared to either the α-tubulin or lamin B1 standards. Results are expressed as fold increase *vs* control acini.

2.6. Analysis of CCL2 mRNA by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from pancreatic acini using the RNAeasy kit treated with amplification grade DNase 1 (Qiagen, Valencia, Spain) according to the manufacturer's instructions. The integrity of RNA based on the presence of well-defined 28S and 18S rRNA bands was assessed in 1% agarose gel and RNA concentration was measured by spectrometry.

For first strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit for RT-qPCR (Bio-Rad Laboratories, Hercules, CA). cDNA was amplified by a polymerase chain reaction (PCR) on a StepOne system (Applied Biosystems, CA, USA) using the IQ SYBR® Green Supermix (Bio-Rad Laboratories) and specific primers (Roche) for each gene: chemokine (C–C motif) ligand 2 (CCL2) (NM_031530) forward: 5′-TAGCATCCACGTGCTGTCTC-3′ reverse: 5′-CCGACTCATTGGGATCATCT-3′ and 18S (NM_046237) forward: 5′-AGTCCCTGCCCTTTGTACACA-3′ and reverse: 5′-GATCCGAG GGCCTCACTAAAC-3′. The cDNA was amplified by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. Amplifications were performed in triplicate and the melting curves were analyzed to validate product specificity. The ratio of the relative expression of target genes to 18S (internal standard) was calculated by using the ΔΔCt method. Results were expressed as fold increase over untreated cells.